PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

V. THE EFFECT OF COPPER ON EXTRA DIETARY ASCORBIC ACID [1]

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Certain food products may contain appreciable quantities of both ascorbic acid and copper. The best example is liver, reported to contain approximately 35 mg. of the vitamin and 2 mg. of copper per 100 gm. (Waisman and Elvehjem, '41). There are also pharmaceutical preparations that offer mixtures of vitamins and minerals including ascorbic acid and copper. A necessary precaution in assaying such products is to minimize the period of contact between the copper and ascorbic acid during the extraction of the material. The incompatability of copper and ascorbic acid in the presence of oxygen is well recognized. This raises the question of what happens in the digestive tract when prolonged contact between the vitamin and copper occurs.

Stotz, Harrer, Schultze and King ('38) reported that ascorbic acid oxidase preparations have no more catalytic activity than a solution of non-specific protein containing an equivalent amount of copper. A mixture of egg albumen and traces of copper was shown by these investigators to simulate all the observed properties of their ascorbic acid oxidase preparation. Some doubt is cast on this interpretation by the thermolability and optical specificity of ascorbic acid oxidase, properties which strongly suggest a specific protein as the catalyst. Nevertheless, there are indications in the literature (Ramasarma and associates, '40; Lovett-Janison and Nelson, '40) that this enzyme may be a copper-protein complex.

Mapson ('41) has employed the catalytic action of copper (approximately 4 μg. per milliliter) on the oxidation of ascorbic acid (100 μg. per milliliter) in relatively pure systems for evaluating the influence of various agents on stabilizing the vitamin. In the absence of a stabilizer, destruction occurred in solutions when the copper concentration ex-

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

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ceeded 0.0015 μg. per milliliter. This small amount of copper was rarely obtainable unless special precautions were taken to eliminate copper as a contaminant of the reagents employed. No attempts were made by Mapson to measure dehydroascorbic acid since the latter was regarded as an oxidation product. However, from the practical standpoint, such data would have been important, since dehydroascorbic acid is biologically active. Fortunately, an earlier investigation by Barron and associates ('36) included such measurements. When the catalytic oxidation of ascorbic acid due to the presence of copper was performed in acid solutions (pH 5.0 or less), the oxidized form could be completely reduced to ascorbic acid by hydrogen sulfide, indicating it to be dehydroascorbic acid. Above pH 5.0 the amount of ascorbic acid recovered gradually decreased, until at pH 7.6 only 4% of the oxidized form could be reconverted to reduced ascorbic acid, the oxidation having gone beyond the dehydro stage.

In the present study of the influence of concomitant ingestion of copper on the availability of ascorbic acid in man, the photometric method of Hochberg, Melnick and Oser ('43) was employed for the determination of both the reduced and dehydro forms of the vitamin. The basal diet and test subjects were the same as those previously employed (Melnick, Hochberg and Oser, '45).

EXPERIMENTAL PART

In the preliminary in vitro studies measurements were made of both reduced and dehydroascorbic acid. However, in the case of the urine samples obtained from the test subjects, only total ascorbic acid was determined. In a previous investigation (Hochberg, Melnick and Oser, '45) dealing with the influence of dietary ascorbic acid oxidase on the availability of the vitamin, the urinary figures were partitioned into reduced and dehydroascorbic acid. No differences were observed in the ratio of one to the other during control and test periods despite the demonstrated effectiveness of the oxidase in converting reduced to dehydroascorbic acid prior to absorption. These findings and those reported by Johnson and Zilva ('34) indicated that nothing was to be gained by partitioning urinary ascorbic acid. The latter investigators found no difference in the form and amount of ascorbic acid excreted in the urine following test doses of reduced or dehydroascorbic acid. More recently Berryman and associates ('44) concluded that, although approximately 18% of the total ascorbic acid excreted following dosage with the reduced form of the vitamin is dehydroascorbic acid, this fraction remains relatively constant so that the measurement of either

reduced or total ascorbic acid is satisfactory in short (6-hour) experiments dealing with test dose responses. In the present studies, involving analyses of 24-hour urine samples during the collection of which opportunities exist for the partial and variable conversion of reduced into dehydroascorbic acid (Hochberg, Melnick and Oser, '43), it was considered advisable to measure total rather than only reduced ascorbic acid.

TABLE 1

In vitro tests of the incompatability of copper and ascorbic acid in the basal diet [1] before and after heat inactivation of the enzymes

EXPERIMENT		ASCORBIC ACID FOUND IN DIET		
No.	Condition[2]	Reduced	Dehydro	Total
		mg	*mg*	*mg*
I	Blanched,[3] homogenized basal diet	101	13	114
II	I incubated 6 hrs. at 37°C.	65	27	92
III	I + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	34	38	72
IV	I + 200 mg. of ascorbic acid, incubated 6 hrs. at 37°C.	240	39	279
V	I + 200 mg. of ascorbic acid + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	230	34	264
VI	Unblanched, homogenized basal diet	0	112	112
VII	VI incubated 6 hrs. at 37°C.	1	44	45
VIII	VI + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	1	35	36
IX	VI + 200 mg. of ascorbic acid, incubated 6 hrs. at 37°C.	128	78	206
X	VI + 200 mg. of ascorbic acid + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	134	64	198

[1] 6.5 mg. of copper were found to be naturally present in the diet.

[2] The homogenized mixtures were incubated at their natural pH 5.5 in open beakers and stirred every half hour.

[3] The solid items in the ration were subdivided into small particles (about 1 cu. in.) and dropped immediately into boiling milk (previously deaerated) under an atmosphere of nitrogen. The boiling was continued for 20 minutes. The mixture was then cooled in an ice bath and homogenized under nitrogen.

[4] Copper sulfate was used.

In vitro tests

In table 1 are presented the results of in vitro experiments on the incompatability of copper and ascorbic acid. Two series of tests were conducted, both on the homogenized basal diet.[2] For one series, the foods [3] were first blanched in order to inactivate all enzymes present;

[2] The ration included bread, butter, steak, milk, eggs, sugar, orange, banana, apple, lettuce, tomato and cooked carrots, beets and potatoes.

[3] The solid items in the ration were subdivided in small particles (about 1 cu. in.) and dropped immediately into boiling milk previously deaerated by a stream of nitrogen. The boiling was continued for 20 minutes. The mixture was then cooled in an ice bath and homogenized in a Waring Blendor under nitrogen. In the case of the unblanched diet, the homogenization was carried out under air.

for the other, the unblanched homogenized ration was employed. Aliquots of the dietary mixtures were then maintained for a period of 6 hours at 37°C. at their natural pH of 5.5 in open beakers and gently stirred every ½ hour. The experiments were repeated but this time 7 mg. of copper, as copper sulfate, were added to the daily ration.[4] To other aliquots of the dietary, the equivalent of 200 mg. of ascorbic acid was added, with and without the additional 7 mg. of copper.

The results of the tests indicate that during the incubation of a dietary mixture in which all enzymes have been heat-inactivated there was a small but appreciable loss of ascorbic acid. That this was due to oxidation may be inferred from the fact that the dehydroascorbic acid value had increased. In the presence of added copper this oxidative loss, involving conversion of reduced to dehydroascorbic acid, progressed somewhat further. That these losses in the ascorbic acid appear to be absolute, was shown by the experiments in which added ascorbic acid was quantitatively recovered in the reduced state from the dietary mixture (compare experiments IV and V with experiments II and III, respectively). These tests on the blanched homogenized basal diet are significant since the effect of copper per se was measured; no active enzymes or native protein were present to complicate the interpretation.

Simple homogenization of the unblanched homogenized basal diet resulted in marked oxidation of the ascorbic acid, practically all of it going to but not beyond the dehydroascorbic stage. However, during the period of incubation of the dietary mixture at 37°C. fully 60% of the dehydroascorbic acid was further oxidized. In the presence of added copper there was a small additional loss of dehydroascorbic acid. Extra ascorbic acid added to the mixture was mostly oxidized to and some even beyond the dehydroascorbic acid stage. Additional copper caused no more marked destruction of the ascorbic acid. In this series of experiments the decreases in ascorbic acid content of the dietary mixtures were due principally to the action of ascorbic acid oxidase. This enzyme is believed (Green, '41) to catalyze the oxidation of ascorbic acid in an absolute manner, the rate of oxidation being independent of the concentration of the vitamin. The present results on the fate of extra ascorbic acid added to the incubated dietary mixtures confirm this hypothesis. The findings with added ascorbic acid and added copper, however, fail to support the suggestion advanced by Stotz and associates ('38) that ascorbic acid oxidase is a non-specific protein-copper complex. If this were the case, an increase in the oxidative destruction of ascorbic

[4] 6.5 mg. of copper were found by analysis (McFarlane, '32) to be naturally present in the 2500 gm. of dietary mixture; this was equivalent to 2.6 μg. of copper per ml. of mash.

acid should have occurred when additional quantities of copper were added to the unblanched homogenized dietary mixture containing appreciable amounts of native protein. This was not noted.

In the present series of experiments the dietary mash contained approximately 2.6 μg. of copper per milliliter. The addition of the 7 mg. of extra copper to the whole day's ration increased the copper concentration to approximately 5.4 μg. of copper per milliliter. Despite the fact that this concentration is greater than the 4.0 μg. of copper per milliliter, employed by Mapson ('41) in his experiments dealing with factors affecting the stability of ascorbic acid in pure systems, much smaller losses of ascorbic acid were observed. It is to be noted that Mapson kept the test solutions at 100°C. though for a period only one-twelfth of that employed in the present investigation. It may very well be that in the pure systems employed by Mapson much greater destruction of the vitamin occurred primarily because of the absence of natural protective agents. It is known that in natural extracts there are present various substances, such as glutathione and other sulfhydryl compounds, which are capable of protecting the vitamin by their preferential oxidation. Such factors were undoubtedly operative in the present study.

In vivo test

The physiological availability study involved measurements of the extra urinary excretion of ascorbic acid by human subjects receiving a constant basal diet and taking a 200-mg. test dose of extra ascorbic acid in pure solution, at one period alone, and the other followed by a solution of copper sulfate. The amount of copper in the test dose was 7.0 mg. The ratio of added ascorbic acid to added copper in both the in vitro and in vivo tests (200:7) is the same as the ratio, but about seven times the amount, of the minimum daily requirements for these factors.

The results of the availability studies are summarized in table 2. These indicate that no destruction of ascorbic acid occurred in the digestive system as the result of taking copper along with the vitamin in the doses indicated. These results were in good agreement with the data obtained in the in vitro test. It may be emphasized that these studies relate to the effect of copper ingestion on the fate of extra dietary ascorbic acid; whether prolonged ingestion of copper would depress the basal excretion (i.e., of vitamin from the diet itself) remains to be demonstrated.

TABLE 2

Availability of ascorbic acid in presence of copper.
(All values in milligrams per 24 hours.)

SUBJECT	CONTROL PERIOD: NO COPPER, 200 MG. ASCORBIC ACID			TEST PERIOD: 7.0 MG. COPPER, 200 MG. ASCORBIC ACID		
	Basal excretion	After test dose	Per cent of test dose excreted	Basal excretion	After test dose	Per cent of test dose excreted
J.C.	57	183	63	23	130	54
E.M.	41	159	59	17	130	57
D.M.	47	131	42	30	150	50
M.H.	70	162	46	24	152	64
Average	54	159	53	29	141	56

Availability of ascorbic acid in presence of added copper $= \frac{56}{53} \times 100 = 106\%$.

SUMMARY

The addition of 7 mg. of copper to a homogenized mixture of a day's ration and incubation of this mixture for a period of 6 hours at 37°C. resulted in a small but real destruction of the ascorbic acid present. Since no more vitamin was destroyed in the presence of added ascorbic acid (200 mg.), this destruction is regarded as absolute. The factor primarily responsible for the marked loss of ascorbic acid in a homogenized dietary mixture is ascorbic acid oxidase. The addition of extra copper to such a mash (unblanched) failed to produce markedly greater destruction of vitamin C. This would seem to indicate that ascorbic acid oxidase is a specific enzyme and not simply a loose complex of copper with protein of unspecific nature. Human availability studies have indicated that in the digestive tract ascorbic acid and copper in the quantities taken, 200 mg. and 7 mg., respectively, are not incompatible.

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PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

VI. THE EFFECT OF ADSORBENTS ON THIAMINE [1]

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The ingestion of various adsorbents for one therapeutic reason or another is not an uncommon practice. However, some of these substances have the capacity to adsorb the B-vitamins and indeed have been employed for the concentration and isolation of these vitamins from natural sources.

Prior to the synthesis of thiamine, the International Standard for vitamin B_1 was a fuller's earth adsorbate of an extract of rice polish. This particular preparation was subsequently shown by Keresztesy and Sampson ('37) to be unsatisfactory as a reference standard. In rat curative tests they found that a quinine sulfate eluate of the clay was fully twice as effective as the direct feeding of the adsorbate to the animal. Apparently the adsorbate contained twice as much thiamine as indicated by the standard assay, but the animal organism could elute only one-half. Despite these findings some pharmaceutical preparations still include fuller's earth adsorbates of the B vitamins.

In 1940 Robinson, Melnick and Field reported that almost without exception patients with peptic ulcers receiving antacid medication excreted very small amounts of thiamine in the urine before and after the taking of a test dose of the vitamin. It was subsequently demonstrated (Melnick, Robinson and Field, '41) by in vitro and in vivo experiments that the antacid medication and not the thiamine content of the diet was at fault. The creation of an alkaline reaction in the gastrointestinal tract favored greater destruction of thiamine, while adsorbents, particularly magnesium trisilicate, caused a loss of thiamine to the organism by rendering the vitamin unavailable for absorption.

Adsorbents have been recommended for the treatment of certain intestinal disorders. Kaolin has long been employed for the control of

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th Meeting of the American Chemical Society, New York, N. Y. The expenses of this study were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

diarrhea when the agent responsible for the condition is capable of being adsorbed, for example, in cases of food poisoning or infection with gastroenterotoxic organisms. Since chronic diarrhea can also be responsible for conditioned malnutrition (Jolliffe, '43), certain pharmaceutical products consisting of mixtures of kaolin and vitamins, particularly those of the B-complex, have been recommended for the treatment of the diarrhea and at the same time for effecting restoration of the vitamin content of the tissues. However, adsorption is not a specific phenomenon. Adsorbing agents, taken orally, can bind nutrients and enzymes, as well as toxic substances present in the gastrointestinal tract. Thus, the possibility exists that the presence of the kaolin may render unavailable not only the vitamins taken with the adsorbent but also nutrients in the dietary which are capable of being adsorbed. This action of adsorbents may be likened to that of mineral oil in reducing the availability of fat-soluble vitamins because of their preferential solubility in the unabsorbable oil and their subsequent excretion in the stools.

In the present study the influence of the concomitant ingestion of fuller's earth and of kaolin on the availability of thiamine to man was investigated. Use was made of the bioassay technic described in a previous paper (Melnick, Hochberg and Oser, '45). The vitamin intake was determined by the thiochrome procedure (Hennessy, '41) and urinary excretion by the colorimetric method (Hochberg and Melnick, '44).

Thiamine was selected as the vitamin for study since in vitro tests demonstrated that it was more readily adsorbed and more firmly retained by the adsorbents than either of the other two major vitamins of the B complex, riboflavin and niacin, for which the assay technic is applicable.

EXPERIMENTAL PART

In vitro tests

That both fuller's earth and kaolin can readily adsorb thiamine from aqueous solution is indicated by the data in table 1. For each experiment a solution containing 5 mg. of thiamine was used. The pH of the suspension was 4.5 and contact between vitamin and adsorbent was for a period of 6 hours at 37°C. The results suggest that fuller's earth may have a greater affinity than kaolin for thiamine.

In vivo tests

Two commercially available products were employed as the test materials in the human availability studies: (a) a B-complex gelatin cap-

sule, containing fuller's earth; and (b) a pharmaceutical preparation of pure kaolin recommended for the control of diarrhea. The adsorbents were the same as those employed in the in vitro tests described above. Each assay involved comparison of the extra urinary excretion of thiamine by the test subjects receiving a uniform, adequate basal diet and taking a test dose (5.0 mg.) of thiamine during the control period, as a pure solution alone; during the test period, along with the adsorbent.

The results of the availability study in which five of the vitamin capsules were taken postprandially by each of the test subjects are presented in table 2. Each vitamin capsule contained 1000 μg. of thiamine

TABLE 1

In vitro adsorption of thiamine from pure aqueous solutions.[1]

ADSORBENT	QUANTITY	THIAMINE ADSORBED
	gm.	%
Fuller's earth	0.25	99
	0.50	100
	2.00	100
Kaolin	0.5	54
	1.0	73
	100.0	100

[1] In each experiment the adsorbent was added to an acetate-buffered (pH 4.5) solution containing 5 mg. of thiamine. Contact between vitamin and adsorbent was at 37°C. for a period of 6 hours. The total volume was 30 ml. in every case except in the last instance where the 100 gm. of kaolin were added to 1500 ml. of solution.

TABLE 2

Availability to man of thiamine in B-complex capsule containing fuller's earth.

SUBJECT	CONTROL PERIOD: PURE SOLUTION DOSE, 5.0 MG. THIAMINE			TEST PERIOD: 5 CAPSULE[1] DOSE, 5.0 MG. THIAMINE		
	Basal excretion	After test dose	Test dose excreted	Basal excretion	After test dose	Test dose excreted
	mg./24 hours		%	*mg./24 hours*		%
J.C.	0.20	1.08	18	0.24	0.41	3
E.M.	0.23	1.00	15	0.27	1.08	16
D.M.	0.30	1.35	21	0.29	0.58	6
M.H.	0.26	1.50	25	0.21	0.59	8
H.H.	0.25	1.39	23	0.23	0.48	5
Average[2]	0.25	1.33	22	0.24	0.52	6

Availability of thiamine in capsules $= \frac{6}{22} \times 100 = 27\%$.

[1] Each containing 333 mg. of fuller's earth as a riboflavin adsorbate.

[2] Omitting subject E.M.

and 500 of riboflavin, the latter adsorbed to 333 mg. of fuller's earth.[2] However, the available thiamine content of these capsules was found to be only 27% of the total, as indicated by the four subjects showing diminished excretion of the test dose.[3]

It is of interest to note that one subject was able to elute the thiamine completely from the fuller's earth adsorbate. The test was repeated with this individual and essentially the same results were obtained. This variability in the responses of the human subjects finds its counterpart in the variability in the responses of rats in the old biological assay procedure when the International Standard was employed as the reference material.

In order to ascertain whether the apparent low availability of thiamine was due simply to a slow rate of absorption of the vitamin, evidenced by a delay in its excretion, analyses were conducted on the urine samples collected during the second 24-hour period following dosage. The "carry-over" values for each period were found to be small, in each case about one-sixth of the extra urinary excretion figures for the first 24 hours following dosage. Since these carry-over values bore the same relationship to each other as the figures for the first 24-hour samples, delayed absorption could not have been responsible for the low thiamine availability.[4]

A similar study was conducted to determine the influence of the concomitant ingestion of kaolin on the availability of thiamine. In the control of diarrhea it is the practice to give kaolin in doses of from 50 to 100 gm. at 3-hour intervals (Goodman and Gilman, '41). Smaller doses of 5 to 10 gm. are frequently taken daily over long periods of time to

[2] Thus, the thiamine was incorporated in the mix prior to encapsulation as the free crystalline material. However, during the disintegration of the tablet in vivo adsorption of the thiamine undoubtedly occurs due to the favorable pH of the gastric contents. Indeed, even when this product was assayed chemically, interference in the extraction of the thiamine was encountered. A value of 688 μg. of thiamine per capsule was obtained by the standard thiochrome procedure (U. S. Pharmacopoeia XII, '42). Only after exhaustive strong acid (2N HCl) extraction could the claimed quantity of thiamine (1000 μg.) be found present. This led to the suspicion that the vitamin in this capsule would only be partly available to man.

[3] Similar studies indicated that only 79% of the riboflavin present in these capsules was available. In vitro tests also indicated that the riboflavin could be more easily extracted from the adsorbent than thiamine.

[4] In other related studies conducted in these laboratories analyses were conducted on the urine samples collected during the second as well as the first 24-hour period following dosage. In no case did the carry-over values (second 24-hour exeretions) alter the interpretation of the data based solely on the figures obtained for the first 24-hour samples. If vitamin absorption from the gastrointestinal tract is to be adequate, it should occur well within the first 24 hours after dosage; otherwise the vitamins are excreted in the stools and thereby lost to the organism.

regulate bowel action. In the present study a single dose of 100 gm. of kaolin in aqueous suspension was taken postprandially by each subject. The test dose of thiamine followed immediately after the kaolin suspension had been taken. The thiamine was dissolved in milk rather than in water as had been customary. This was intended to provide a longer period of contact in the stomach between the kaolin and the thiamine, thereby favoring adsorption of a greater portion of the dose. Furthermore, the design of the present experiment more nearly duplicated conditions in practice with respect to the action of the adsorbent on dietary thiamine.

TABLE 3

Availability of thiamine in presence of kaolin.

SUBJECT	CONTROL PERIOD: PURE SOLUTION DOSE, 5.0 MG. THIAMINE [1]			TEST PERIOD: 100 GM. KAOLIN FOLLOWED BY 5.0 MG. THIAMINE IN SOLUTION		
	Basal excretion	After test dose	Test dose excreted	Basal excretion	After test dose	Test dose excreted
	mg./24 hours		%	*mg./24 hours*		%
J.C.	0.20	1.45	25	0.20	1.56	27
E.M.	0.23	0.89	13	0.24	1.48	25
D.M.	0.30	1.13	17	0.29	1.40	22
M.H.	0.26	1.17	18	0.23	1.58	27
H.H.	0.24	1.20	19	0.26	1.23	19
Average	0.25	1.17	18	0.24	1.45	24

Availability of thiamine in presence of kaolin $= \frac{24}{18} \times 100 = 133\%$.

[1] Thiamine taken dissolved in milk.

The results of the urinary excretion tests following dosage with kaolin and extra thiamine are presented in table 3. These indicate that kaolin, unlike fuller's earth, does not diminish the availability of thiamine. The percentage excretion of the test dose following the taking of the vitamin along with kaolin was not less than that noted when the pure solution of thiamine was taken alone. In vitro tests (see table 1) have indicated that kaolin adsorbs the vitamin quite readily and the pH of the gastric contents is optimal for this adsorption. Apparently, at the more alkaline pH of the small intestine, the thiamine is effectively eluted from the kaolin adsorbate. The greater-than theoretical availability of the vitamin, noted in three of the five subjects, is real and not due to experimental error. In some experiments which have been reported (Oser, Melnick and Hochberg, '45) and in others which are in progress in our laboratories, it has become evident that thiamine shows

a variable degree of stability in the gastrointestinal tract prior to absorption and that the vitamin may be protected by the concomitant ingestion of various materials.

SUMMARY AND CONCLUSIONS

Certain substances, capable of adsorbing the B vitamins, are taken regularly and in appreciable quantities over long periods of time for the treatment of various clinical disorders. Human availability studies have been conducted to determine the influence of the concomitant ingestion of two such adsorbents, fuller's earth and kaolin, on the availability of thiamine. These materials were shown to adsorb the vitamin very readily from aqueous solution. Whereas the availability of thiamine was markedly reduced by the fuller's earth, no interference occurred when kaolin was taken along with thiamine. Indeed, the latter adsorbent appeared to protect the vitamin during its passage through the gastrointestinal tract so that a greater-than-theoretical value for available thiamine was obtained. In vitro tests have not proved satisfactory for predicting the influence of an adsorbent on vitamin availability. The present experiments cast doubt upon the wisdom of taking continuous large doses of adsorbing agents, unless precautions are taken to insure sufficient vitamin intake.

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EFFECT OF SOYBEAN PHOSPHATIDES ON VITAMIN A METABOLISM

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TWO FIGURES

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We have previously reported that commercial soybean lecithin contains an unknown factor which influences carotene and vitamin A utilization in the rat (Slanetz and Scharf, '43). As pointed out in this report, our diet differed from that of other investigators insofar as we substituted the synthetic members of the B complex for yeast as a supply of the B vitamins known to be essential for normal growth in the rat.

Patrick and Morgan ('43) confirmed our findings in the chick and also demonstrated that the unknown factor may be extracted from yeast by suitable solvents. Earlier, Polskin ('40) had reported about the effect of lecithin on the utilization of vitamin A in the chick and found better growth response and an increased liver storage of vitamin A when lecithin was added to the diet. No difference in total liver lipids could be noted between the controls and the lecithin-treated animals.

Adlersberg and Sobotka ('43) reported elevated vitamin A blood levels in man after ingestion of soybean lecithin.

Jensen, Hickman and Harris ('43), however, questioned our findings because our soybean lecithin preparation contained vitamin E and they referred the observed effect to the presence of this vitamin. However, the diet in their experiment contained 8% yeast and, therefore, their experimental conditions were not comparable to ours.

In a recent report (Scharf and Slanetz, '44) we proved that vitamin E is not the factor responsible for our observations and while this report was in the printer's hands Patrick and Morgan ('44) published their experiments which also proved that vitamin E was not responsible for the reported action of soybean lecithin. As a matter of fact, Patrick and Morgan as well as we, found indications of vitamin E deficiency in the animals in spite of sufficient amounts of vitamin E in the diet.

In our previous report (Slanetz and Scharf, '43) we had mentioned the well known relation between soybean cephalin, contained in our commercial soybean lecithin, and vitamin E concerning its antioxidant effectiveness. Patrick and Morgan ('44) also seemed to be inclined to explain the observed effects as the stabilizing influence of soybean cephalin on vitamin E and indirectly on vitamin A.

Since our previous work (Slanetz and Scharf, '43) indicated that soybean lecithin influences utilization of vitamin A when borderline amounts of vitamin A are fed to rats, we felt it advisable to study the effect of soya lecithin on vitamin A utilization when larger amounts of vitamin A are fed and its effect on storage of vitamin A in the liver. In this report we present our findings on the above as well as the results of our study on the influence of heating, halogenation, and other factors on the unknown factor in soybean lecithin.

EXPERIMENTAL

Female albino rats, Sherman strain, from our laboratory colony, weighing 90–110 gm. at the start of the experiment were kept in individual metal cages equipped with screen floors. The rats were weighed weekly. Food consumption was determined at intervals. The percentage composition of the basal diet was as follows: vitamin-free casein (Labco) 20; cerelose 72; Sure's no. 1 salts 4; corn oil 2; wheat germ oil 2; viosterol 0.002. The diet was supplemented with thiamine 2 mg., riboflavin 4 mg., pyridoxine 4 mg., nicotinic acid 100 mg., vitamin K 4 mg., inositol 1000 mg. and para-aminobenzoic acid 300 mg., per kilogram of diet. Synthetic calcium pantothenate (100 μg.) was given orally daily. Each rat was given orally 30 μg. of carotene daily. The purified diets and mixtures were freshly prepared every 2 weeks and stored at 4°C.

Forty-eight rats were distributed equally among eight groups and the groups fed as follows: basal diet only; and basal diet supplemented daily, respectively, with 3 mg. choline, 1% soybean lecithin, 1% heated soybean lecithin, 5% cholesterol, 1% iodized lecithin, 3% iodized lecithin, and 0.3% iodine in the form of potassium iodide. After 10 and 12 weeks on the diet several rats from each group were sacrificed and vitamin A determinations made on the blood and liver.

DISCUSSION

Commercial soybean lecithin is an extract of soybean lipids and contains about 30–35% soybean oil and about 65% soya phosphatides. There are also present small amounts of carbohydrates, phytosterols, and traces of other as yet unidentified substances. The soya phospha-

tides comprise soya lecithin, soya cephalin, and lipositol (inositol phosphatides) which are present in approximately equal amounts. Separation and purification of the various phosphatides and substances present in soybean lecithin has as yet not been successful and even the most purified fractions have been found to be contaminated to the extent of 10–20% with other substances. Commercial soybean lecithin

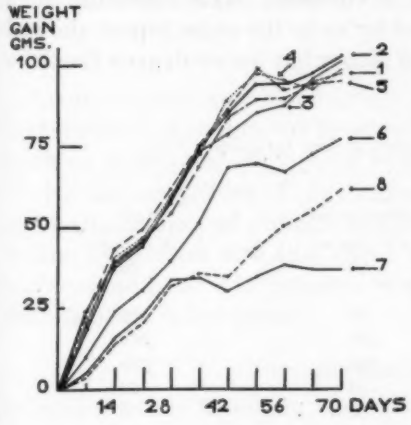

Figure 1

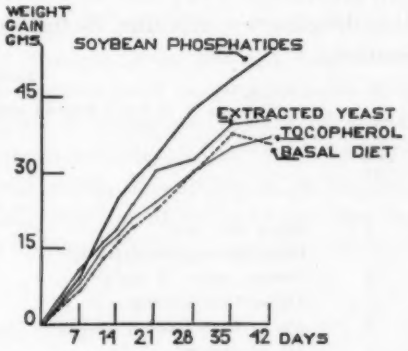

Figure 2

Fig. 1 Influence of various dietary supplements on the growth of rats. Group 1 was fed the basal diet only. The other groups received the basal diet supplemented as follows: group 2, 3 mg. choline daily; group 3, 1% soybean lecithin; group 4, 1% heated soybean lecithin; group 5, 5% cholesterol; group 6, 1% iodized lecithin; group 7, 3% iodized lecithin; group 8, 0.3% iodine in the form of potassium iodide.

Fig. 2 Influence of soybean phosphatides, ether-extracted yeast and tocopherol on weight response of rats.

is devoid of vitamin A but does contain about 1.2–1.4 mg. vitamin E per gram as we have reported before (Scharf and Slanetz, '44). We, as well as Patrick and Morgan, have proved that the vitamin E content of the soybean lecithin used is not responsible for the observed effects (fig. 2). Therefore, the presence of an unknown factor in commercial soybean lecithin that affects vitamin A storage in the liver is indicated by our findings.

Figure 1 shows that rats receiving an ample supply of vitamin A do not show any marked difference in growth response whether given soybean lecithin or not. However, there was a marked increase of vitamin A in the blood as well as a very striking elevation of the liver vitamin A when the commercial lecithin was fed (table 1). Figure 1 also shows that addition of choline did not influence rate of growth.

The presence or absence of choline in our diet did not seem to influence either blood or liver vitamin A, nor did cholesterol. No gross pathology was observed in the livers, indicating a sufficient supply of the lipotropic factors, choline and inositol, in our diet.

Patrick and Morgan ('43) reported that a deficiency of the unknown factor could be developed in chicks receiving yeast extracted by fat solvents as a source of the vitamin B complex. Rats receiving such extracted yeast and a diet as described by us in the same report showed also deficiency symptoms, though to a somewhat lesser degree than the controls.

TABLE 1

Vitamin A in blood plasma and liver of rats fed various diets.

GROUP NO.	DIET	VITAMIN A	
		Blood plasma	Liver
		I.U./100 ml.	*I.U./100 gm.*
1	Basal diet only	93	20
	Basal diet supplemented by:		
2	Choline daily (3 mg.)	88	20
3	1% soybean lecithin	150	350
4	1% soybean lecithin heated	120	200
5	5% cholesterol	99	20
6	1% iodized lecithin	57	20
7	3% iodized lecithin	27	20
8	0.3% iodine (KI)	111	30

As it is known that heating lecithin to higher temperatures or halogenation of lecithin does not interfere with its antioxidant effect in vitro, iodized soybean lecithin as well as soybean lecithin[1] heated to 110°C. for 1 hour was fed to the rats. The results show that the heating of the soybean lecithin did not destroy its effectiveness but diminished it. On a diet as described in our previous experiment (Scharf and Slanetz, '44) heated lecithin showed a lesser effect on growth than unheated lecithin.

The incorporation of 10% iodine in soybean lecithin[2] apparently destroyed the effectiveness of the unknown factor. However, the rats receiving iodine in form of iodized lecithin or potassium-iodide also showed impaired growth. These results require further investigation.

The results described above do not indicate that the reported effect is identical with the antioxidant effect of soybean cephalin on vitamin

[1] We are indebted to the American Lecithin Company for supply of commercial soybean lecithin.

[2] We are indebted to Dr. P. L. Julian and Mr. H. Iveson of the Glidden Co., Chicago, Ill., for the iodized lecithin

E. Polskin ('40) observed that chicks receiving lecithin and vitamin A and having an elevated vitamin A content of the liver lose much less of their vitamin A reserve in a given time when put on a vitamin A deficient diet than those that receive no lecithin. This, likewise, is hard to explain as an antioxidant effect. Further experiments may determine whether the observed effect is due to the antioxidant action of cephalin or whether an unknown nutritional factor plays a role.

SUMMARY

Liver storage and blood levels of vitamin A in the rat have been investigated as influenced by specific factors used as supplements in a purified basal diet containing synthetic B vitamins instead of yeast. Under the conditions of our experiment commercial soybean lecithin markedly influenced storage and blood levels of vitamin A in the rat. Iodine interfered with this effect while heating of soybean lecithin interfered but little. The presence of an unknown factor in commercial soybean lecithin is indicated.

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NUTRITIVE VALUE OF DISTILLERS' DRIED SOLUBLES AS A SOURCE OF PROTEIN [1, 2]

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Distillers' dried solubles has recently assumed importance as an ingredient in commercial feeds for poultry and farm animals. Although distillers' solubles was a waste product prior to 1939, the production of dried solubles has increased rapidly during recent years. This product has been shown to be a valuable vitamin supplement (Bauernfeind et al., '44); Boruff et al., '40; Synold et al., '43). Because of its high protein content it also comes into consideration as a protein supplement. However, its value as a protein supplement has not received extensive study. Sloan ('41) found that it could be used satisfactorily to supply approximately 12% of the total protein in poultry rations. Bauernfeind et al. ('44) suggest its use as a supplement to soybean meal since it contains 2.7% methionine. Joseph et al. ('42, '43) reported that the amino acid composition of dried solubles resembled that of yeast and found that both the protein content and palatability could be improved by growing yeast in the stillage. The nutritive value of yeast has been reviewed by Carter and Phillips ('44).

In the present investigation the nutritive value of distillers' dried solubles is evaluated by biological and chemical means.

EXPERIMENTAL

The dried solubles used in this study was derived from a mash containing 51% corn, 42.5% wheat, and 6.5% barley malt. The thin sillage was concentrated in multiple-effect evaporators under reduced pressures at temperatures ranging from 130° to 230°F. to a thick syrup of approximately 30% solids and then dried on drum driers. The protein content of the moisture free product was 37.14%. It contained proteins derived from these cereal grains and yeast.

[1] Journal Paper no. 187 of the Purdue University Agricultural Experiment Station.

[2] This investigation was supported in part by a grant from Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

The nutritive value of dried solubles was investigated by means of biological tests with albino rats as the experimental animal. In each series of experiments hydrogenated vegetable oil was used to adjust the rations to the same fat content so that differences in energy values should not be a factor affecting food consumption. In addition to the mixed rations, vitamin supplements containing sufficient amounts of all vitamins for normal growth, independent of the vitamin content of the rations were fed 3 times weekly in separate containers. The vitamin B complex supplement contained 200 mg. riboflavin, 2 gm. nicotinic acid, 100 mg. pyridoxine hydrochloride, 100 mg. thiamine chloride, 2 gm. calcium pantothenate, 30 gm. choline chloride, 300 gm. "Labco" rice polish concentrate no. II, and 10 gm. sodium chloride per liter. The cod liver oil contained not less than 1500 U.S.P. units of vitamin A and 100 U.S.P. units of vitamin D per gram. The amounts fed were equivalent to 27 mg. of cod liver oil and ½ ml. of vitamin B complex supplement per day.

Distillers' dried solubles as a sole source of protein

The growth value of dried solubles as a sole source of protein was determined and compared with values for corn and casein. Since casein is known to produce optimum growth at a 15% protein level, all comparisons were made at this level. In this trial ten rats, five male and five female, of an average weight of 42 gm. were placed on experiment. They were fed ad libitum for a period of 6 weeks. A record was kept of the weekly weight and food consumption of the rats. The composition of the rations and the results are given in table 1. The proteins of dried solubles (lot 2) were found to be more deficient than the proteins of corn (lot 1). In fact the proteins of dried solubles were so deficient that they contributed little above maintenance requirements. In contrast to the response with casein (lot 4) it is evident that the proteins of both dried solubles and corn are critically deficient.

Distillers' dried solubles as a supplement to corn

Two series of experiments were conducted in which dried solubles was compared with casein as a supplement to corn. The supplements constituted one-third, one-half and two-thirds of the total protein (15%). Because the growth responses of all lots within each series were almost identical, only one group from each series is reported (lots 5 and 7). Since combinations of dried solubles and corn gave poorer growth responses than corn alone (lot 1), it is apparent that the proteins of dried

TABLE 1

"Distillers' dried solubles" as a sole source of protein and as a supplement to corn, and the effect of fortification with dried brewers yeast.

LOT	1	2	3	4	5	6	7	8	9	10	11	12	13
Ration Ingredients (%)													
Yellow corn	54.5				54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5
Corn gluten meal	23.6							11.8	11.8	11.8	11.8	5.9	
Dried solubles		44.7			29.8			14.9	11.9	8.9	7.5	11.2	14.9
Dried yeast			30.8			20.5			2.1	4.1	5.1	7.7	10.3
Casein				17.5			11.7						
Dextrin	16.4	50.3	61.4	74.5	11.8	19.3	27.9	14.1	14.8	15.6	16.0	15.7	15.5
Hydrogenated vegetable oil	2.5	2.0	4.8	5.0	0.9	2.7	2.9	1.7	1.9	2.1	2.1	2.0	1.8
McCollum salts no. 185	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Per cent of total protein from													
Corn	100				33.4	33.4	33.4	66.7	66.7	66.7	66.7	50.0	33.4
Dried solubles		100			66.6			33.3	26.7	20.0	16.7	25.0	33.3
Dried yeast			100			66.6			6.6	13.3	16.6	25.0	33.3
Casein				100			66.6						
Rats — number	10	10	10	10	10	10	10	10	10	10	10	10	10
Average gain (gm.) — 6 wks.[1]	67	26	68	189	55	138	191	54	87	104	119	123	144
Feed efficiency	5.14	8.09	4.82	3.08	5.4	3.57	3.20	5.58	4.51	4.18	3.91	3.91	3.73

[1] The standard error of the *difference* between any two average gains is 8.0 gm. Consequently the difference necessary for significance is 16 gm. at the 5% level or 21 gm. at the 1% level.

solubles do not supplement those of corn. This is emphasized by comparison with lot 7.

The effect of fortification of distillers' dried solubles with dried brewers' yeast

Since Joseph et al. ('43) found that the protein content of stillage derived from corn mashes could be increased by the growth of yeast in the product, studies were made to determine the feasibility from a nutritional viewpoint of increasing the yeast protein content of distillers' dried solubles. To simulate a product containing more yeast protein, dried solubles was used in combination with dried brewers' yeast.[3] Dried brewers' yeast and combinations of dried solubles and dried brewers' yeast were compared as supplements to corn. Although yeast (lot 3) was no better than corn (lot 1) as the sole source of protein, it effectively supplemented corn (lot 6). When combinations of yeast and dried solubles were used as supplements to corn, it was found that the supplemental value increased progressively with the amount of yeast in the ration (lots 8–13). Maximum supplemental action was obtained in the ration of lot 13 which contained equal amounts of yeast, dried solubles and corn protein. No advantage would be gained by further increasing the yeast content as twice this amount of yeast did not supplement corn any better (lot 6). These results demonstrate that it would be nutritionally valuable to increase the yeast content of dried solubles.

"Biological value" determinations

Nitrogen metabolism studies were conducted to more critically evaluate dried solubles as a sole source of protein and as a protein supplement. Biological values were determined according to the method of Mitchell and Carman ('26) for dried solubles, dried brewers' yeast, corn, casein, a combination of dried solubles and corn, and a combination of dried solubles and yeast. The combinations were used to investigate supplemental action between these sources of protein. Two male and three female rats of approximately 150 gm. weight were used in each determination. The experiment was divided into five periods of 11 days each. The first and last periods were used as standardization periods to obtain data for calculating the endogenous and metabolic nitrogen for the intervening test periods. During the standardiza-

[3] Fleischmann's Irradiated Dry Yeast obtained from Standard Brands, Inc., New York, N. Y.

tion periods, a ration containing dried whole eggs at a 4% protein level was fed, as described by Mitchell and Carman ('26) and during the test periods rations containing specific proteins at a level of 10% were fed. The first 4 days of each test period were used to adjust the rats to the new rations and for the elimination of any food other than the new test ration from the digestive tract. The urine and feces were collected during the last 7 days of each period and analyzed for total nitrogen. The composition of the rations used in the determinations and the biological values obtained are given in table 2.

It was found that biological values for dried solubles, corn, and the combination of dried solubles and corn were relatively low in comparison to the values for yeast, casein, and the combination of yeast and dried solubles. The biological value for a combination of proteins

TABLE 2

The evaluation of the proteins of distillers' dried solubles by means of "biological value" determination.

	LOW-EGG NITROGEN	DRIED SOLUBLES	DRIED SOLUBLES DRIED YEAST	DRIED YEAST	CORN	CORN DRIED SOLUBLES	CASEIN
Ration ingredients (%)							
Yellow corn					49.0	24.5	
Corn gluten meal					12.0	6.0	
Dried whole eggs	8.5						
Dried solubles		29.8	14.9			14.9	
Dried yeast			10.3	20.5			
Casein							11.7
Sucrose	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cellu-flour [1]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Dextrin	54.5	32.2	35.9	39.7	1.1	16.6	48.3
Hydrogenated vegetable oil	19.0	20.0	20.9	21.8	19.9	20.0	22.0
McCollum salts no. 185	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Total protein in ration (%) from	4.0	10.0	10.0	10.0	10.0	10.0	10.0
Corn (%)					100.0	50.0	
Dried whole eggs (%)	100.0						
Dried solubles (%)		100.0	50.0			50.0	
Dried yeast (%)			50.0	100.0			
Casein (%)							100.0
Biological value [2]		53	64	61	49	50	65
Estimated biological value [3]			57			51	
Digestibility coefficient		84	82	83	93	88	100

[1] Obtained from Chicago Dietetic Supply House, Chicago, Illinois.

[2] The standard error of the *difference* between any two biological values is 3.5. Consequently the difference necessary for significance is 7 at the 5% level and 10 at the 1% level.

[3] Estimated value if no supplemental effect exists.

may be estimated, assuming the absence of supplementation, from the average of the biological values obtained when the proteins are fed separately. In the case of the combination of yeast and dried solubles, the experimental value is greater than the estimated value, which indicates that the proteins of yeast supplement the proteins of dried solubles. The estimated value and the experimental value for the combination of dried solubles and corn are similar which shows that the proteins of dried solubles do not supplement those of corn.

Digestibility coefficients, corrected for the metabolic nitrogen of the feces were also obtained from the data used in calculating the biological value of proteins, and are given in table 2. It is apparent that the proteins of corn and casein are more easily digested than are the proteins of dried solubles and dried brewers' yeast.

Amino acid deficiencies of distillers' dried solubles

The amino acid deficiencies of dried solubles at different protein levels were ascertained by supplementation with essential amino acids. The amounts of amino acids added to the rations were based upon the recommendation of Rose ('37). Since in preliminary experiments additions of half the recommended quantities of the amino acids gave essentially the same response as full amounts, the lower level was used in these studies. In the following experiments only male rats were used and the test period was 18 days (table 3).

As positive and negative controls, casein (lot 14) and dried solubles (lot 15) were fed as sole sources of amino acids. Since the supplementing of dried solubles with all the essential amino acids plus cystine (lot 16) gave no better response than when but seven amino acids were added (lot 17), this eliminated leucine, isoleucine, threonine and valine from further consideration. However, the growth responses were approximately one-half of that obtained with casein (lot 14). This difference in response may be ascribed to differences in the palatability of the rations, if it is assumed that no additional essential amino acids or other growth factors are added by casein.

Since additions of histidine, lysine, and tryptophane (lot 18) or of lysine and tryptophane (lot 19) gave responses similar to those obtained with the addition of eleven or seven amino acids (lots 16, 17), this would indicate that histidine and the other omitted amino acids are not deficient. The omission of lysine (lot 20) definitely inhibited growth, which discloses a severe deficiency of this amino acid, while the response obtained by the omission of tryptophane (lot 21) indicates a less critical deficiency of this amino acid.

Similar tests which were conducted at a 20% protein level showed that this additional amount of protein failed to correct the deficiencies of lysine and tryptophane.

To determine the adequacy of dried solubles in amino acids other than lysine and tryptophane, the protein level of the ration was lowered to 10%. Additions of various combinations of histidine, lysine, and tryptophane to dried solubles were found to be incapable of correcting all the deficiencies at this level of protein, indicating other amino acid deficiencies at this level. The growth response in all cases was limited to little more than maintenance requirements.

TABLE 3

Amino acid deficiencies of distillers' dried solubles when fed at a 15% total protein level.

LOT	14	15	16	17	18	19	20	21
Ration ingredients (%)								
Dried solubles		44.7	44.7	44.7	44.7	44.7	44.7	44.7
Casein	17.5							
Dextrin	74.5	50.3	45.5	47.8	49.5	49.7	50.0	49.6
Hydrogenated vegetable oil	5.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
McCollum salts no. 185	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Amino acids [1]								
1(+) Arginine monohydrochloride			0.1	0.1				
1(−) Cystine			0.3	0.3				
1(+) Histidine monohydrochloride			0.2	0.2	0.2		0.2	0.2
dl Isoleucine			0.5					
1(+) Leucine			0.5					
1(+) Lysine monohydrochloride			0.5	0.5	0.5	0.5		0.5
dl Methionine			0.6	0.6				
dl Phenylalanine			0.7	0.7				
dl Threonine			0.6					
1(−) Tryptophane			0.1	0.1	0.1	0.1	0.1	
dl Valine			0.7					
Per cent of total protein from								
Dried solubles		100.0	100.0	100.0	100.0	100.0	100.0	100.0
Casein	100.0							
Rats — number	5	5	5	5	5	5	5	5
Average gain (gm.) — 18 days [2]	107	13	49	54	46	49	10	40
Feed efficiency	2.32	7.60	2.84	2.89	2.91	3.06	10.18	3.54

[1] All amino acids from Merck and Company, Rahway, N. J., except arginine and cystine which were laboratory preparations.

[2] The standard error of the *difference* between any two average gains is 7.4 gm. Consequently the difference necessary for significance is 15 gm. at the 5% level and 20 gm. at the 1% level.

Since the proteins of dried solubles did not supplement the proteins of corn, experiments were conducted to ascertain the limiting amino acids. The rations contained 15% protein, equal amounts contributed by corn and dried solubles. The amino acid deficiencies were corrected by the addition of 0.1% l (−) tryptophane and 0.5% l (+) lysine, giving an average growth response of 172 gm. in 6 weeks which compared favorably with the response obtained with casein (lot 4). Without supplementation with these amino acids, the gain was only 71 gm. The omission of either lysine or tryptophane impaired growth appreciably. Consequently, dried solubles fails to supplement corn because of deficiencies of both lysine and tryptophane.

It was observed that the growth responses with rations containing dried solubles, even when supplemented with indispensable amino acids, were less than with casein. Since palatability might be a factor influencing the results, a series of paired feeding experiments was conducted. The duration of these experiments was 4 weeks (table 4). When compared at 15% digestible protein levels, dried solubles when supplemented

TABLE 4

Effectiveness of amino acids and combinations of proteins in correcting the amino acid deficiencies of distillers' dried solubles as measured by the controlled food consumption method.

LOT	22	23	24	25	26	27
Ration ingredients (%)						
Yellow corn				46.6	46.6	46.6
Corn gluten meal				2.5	2.5	2.5
Dried solubles	53.5		35.7	17.8	17.8	
Casein		17.5	5.8	5.8		11.7
Dried yeast					12.3	
Dextrin	41.5	74.5	52.9	22.3	15.9	33.1
Hydrogenated vegetable oil	1.4	5.0	2.6	2.0	1.9	3.1
McCollum salts no. 185	3.0	3.0	3.0	3.0	3.0	3.0
Amino acids						
1(+) Lysine monohydrochloride	0.5					
1(−) Tryptophane	0.1					
Per cent total protein from						
Corn				33.3	33.3	33.3
Dried solubles	100.0		66.7	33.4	33.4	
Casein		100.0	33.3	33.3		66.7
Dried yeast					33.3	
Rats — number	6	6	6	6	6	6
Average gain (gm.) — 4 weeks[1]	82	81	79	100	92	104
Feed efficiency	2.79	2.74	2.80	2.70	2.99	2.61

[1] The standard error of the difference between any two average gains is 6.9 gm. Consequently the difference for significance is 14 gm. at the 5% level and 19 gm. at the 1% level.

with lysine and tryptophane (lot 22) gave a growth response comparable to casein alone (lot 23) or to casein and dried solubles (lot 24). This indicates that the addition of lysine and tryptophane corrects all the deficiencies of dried solubles at this protein level. The amino acids added by casein likewise correct the deficiencies of dried solubles, permitting a response comparable to that obtained using casein as the sole source of amino acids.

In a second paired feeding experiment, it was found that a combination of dried solubles and yeast (lot 26) supplemented corn almost as effectively as dried solubles and casein (lot 25) or casein alone (lot 27). This shows that dried solubles may be converted into a good protein supplement for corn by the addition of yeast.

Chemical studies

To further elucidate the nutritive properties of dried solubles and of dried brewers' yeast as a supplement to dried solubles, studies were conducted to determine the amino acid contents as well as to attempt to measure the relative availability of certain amino acids as determined by digestion with proteolytic enzymes.

Fat free samples of dried solubles and dried brewers' yeast, ground to pass a hundred mesh sieve, were analyzed for a number of amino acids. Determinations for dicarboxylic acids, cystine, cysteine, arginine, histidine, lysine, and methionine, were made on acid hydrolyzed samples. As the amount of ammonia nitrogen parallels the amount of dicarboxylic acids present in the protein (Schmidt, '38), values for these amino acids were ascertained from amide nitrogen determinations. Cystine and cysteine were determined on the filtrate from the amide nitrogen determinations after decolorizing with Norite, using Doty's modification ('41a) of procedures developed by Shinohara ('35), Lugg ('32a, b) and Kassel and Brand ('38). Arginine, histidine, and lysine were precipitated with phospho-24-tungstic acid according to the Van Slyke et al. modification ('41a) of Cavett's procedure ('32) and the basic tungstates were decomposed with barium hydroxide. Lysine was estimated by manometric determinations of amino nitrogen and carboxyl carbon in a solution of bases. Carboxyl carbon was estimated by the method of Van Slyke et al. ('41b) as modified by MacFadyen ('42) and amino nitrogen was estimated by the method of Van Slyke ('29). Histidine was determined by the method of Macpherson ('42) and arginine by the procedure of Jorpes and Thoren ('32) as modified by Doty ('41a). In the filtrate from the basic phosphotungstates, phosphotungstic acid was removed by precipitation with barium hydroxide

and methionine was determined by an adaptation of the method of McCarthy and Sullivan ('41). Barium was removed from the solution by precipitation with sulphuric acid before development of color as a slight turbidity was apparent in the colored solutions if appreciable amounts of barium were present. Tryptophane determinations were made on separate samples using an adaptation of Doty's modification ('41b) of the procedures of Bates ('37) and Komm ('26). In addition to the usual blank determinations on the reagents, blank determinations were also made for the slight color which develops as a result of the

TABLE 5

Comparison of relative availability of certain amino acids in distillers' dried solubles and dried brewer's yeast

AMINO ACIDS	DISTILLERS' DRIED SOLUBLES [1]			DRIED BREWERS' YEAST [2]		
	Total	Liberated by enzyme digestion		Total	Liberated by enzyme digestion	
	% of protein	% of protein	% of total	% of protein	% of protein	% of total
Arginine	2.7	0.7	25.9	5.0	0.9	18.0
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0
Cystine	1.0	0.3	30.0	0.4	0.4	100.0
Dicarboxylic acids	23.3	8.3	35.6	15.6	7.9	50.6
Histidine	1.5	0.4	26.7	1.5	0.4	26.7
Lysine	3.5	1.9	54.3	6.2	3.9	62.9
Methionine	1.6	0.8	50.0	1.1	0.5	45.5
Tryptophane	0.5	0.1	20.0	0.7	0.2	28.6
Tyrosine	5.0	1.7	34.0	3.5	1.1	31.4

[1] Contained 40.35% protein (N × 6.25) on moisture and fat free basis.
[2] Contained 51.53% protein (N × 6.25) on moisture and fat free basis.

action of concentrated acid on the samples. Tyrosine was estimated after alkaline hydrolysis by a modification (Doty, '41b) of procedures described by Folin and Marenzi ('29) and Lugg ('37). The KWSZ photometer was used in all colorimetric determinations. The results of the analyses are given in table 5. The values reported are averages of triplicate determinations.

Investigations were conducted to determine the relative availability of these amino acids as measured by their liberation upon digestion with the proteolytic enzymes of pancreatin.[4] An arbitrary period of 24 hours was employed for the digestion of samples and also for the subsequent dialysis. The following digestion procedure was employed.

Add 100 ml. of boiling water to each of a series of samples equivalent to 2 gm. of protein. Maintain temperature at 90°C. for 5 minutes to in-

[4] U.S.P. Pancreatin obtained from Merck and Company, Rahway, N. J.

activate enzymes and to reduce bacterial contamination. Cool and adjust to pH 7.5 with sodium hydroxide. Add 25 ml. of a potassium dihydrogen phosphate-sodium monohydrogen phosphate buffer solution adjusted to pH 7.5. Dilute to 200 ml. and add 2 ml. of pancreatin solution containing 25 mg. of pancreatin per milliliter. Add chloroform, stopper the flask, and digest 24 hours at 37°C. in a constant temperature bath. Remove flasks from the bath, adjust to pH 3.0 with hydrochloric acid and inactivate pancreatin by heating at 90°C. for 5 minutes. Centrifuge to remove insoluble material, concentrate under reduced pressure, and make to volume.

To remove undigested proteins and polypeptides, aliquots of the solutions were dialyzed for 24 hours in a continuous dialyzing apparatus developed by Westfall and Hauge ('41), utilizing a cellophane membrane.[5] The dialysates were concentrated under reduced pressure, made to volume, and analyzed for total nitrogen, using an adaptation of the micro-Kjeldahl method of Ma and Zuzaga ('42). Since successive samples gave dialysates with the same total nitrogen content, composite samples were prepared and concentrated under reduced pressure so that the increased concentration of amino acids would be suitable for analysis. Aliquots were hydrolyzed with HCl (constant boiling mixture) for 6 hours to free any amino acids still present in the form of peptides. Amino acid analyses were made by the methods previously described.

The results of the chemical determination show that yeast protein contains considerably more of the particular amino acids which were found to be deficient in dried solubles protein by biological assays, namely, lysine and triptophane (table 5). The proteins of dried solubles contain only 56% as much lysine and 71% as much tryptophane as is found in yeast protein. Furthermore, the digestion experiments with pancreatin showed that these amino acids were liberated from the proteins of dried solubles to a lesser extent than from yeast. Since it is impossible to simulate the conditions in the digestive tract by in vitro experiments, these values should be considered only as relative indices of the availability of the amino acids. However, as the total amounts of lysine and tryptophane of dried solubles protein as well as the respective percentages liberated were less than that of yeast protein, this offers an explanation of the nutritional limitations of dried solubles, as compared to yeast.

[5] Cellophane dialyzing tubing (19 mm. dia.) obtained from E. H. Sargent and Co., Chicago, Ill.

DISCUSSION

It is recognized that there may be some variation in both the quantity and quality of the proteins in this type of product as prepared by different companies and even by the same company. These variations may be due to the type and proportion of cereal grains constituting the mash, the composition of these grains, the proportionate content of yeast in the final product, and the recovery procedure used.

The possibility of using dried solubles as a protein supplement in the ration of farm animals is limited by its deficiency in certain essential amino acids. By means of biological assays it was ascertained that lysine and tryptophane were the limiting amino acids for growth when this product was fed at a 15% level. The requirements of the animal for these amino acids could not be satisfied by increasing the protein level to 20%. When the protein content was lowered to a 10% level, other amino acid deficiencies became apparent. In studying the supplementing value of dried solubles for corn, it was found that the dried solubles product does not supplement corn because of deficiencies of lysine and tryptophane. Although the lysine and tryptophane content of yeast is higher than that of the cereal grains, the amount of yeast in dried solubles is not adequate to contribute enough of these amino acids to sufficiently enhance the protein quality of this product to make it valuable either as a sole source of protein or as a supplement to corn.

Since Joseph et al. ('43) have previously demonstrated that the protein content of corn stillage could be increased by increasing the yeast content by fermentation methods, a study was made of the nutritional value of dried brewers' yeast and of various combinations of yeast and dried solubles for the purpose of determining the possible role of yeast in the fortification of dried solubles. It was found that yeast was of greater nutritional value than dried solubles when used as a sole source of protein and effectively supplemented dried solubles, corn and combinations of these products. Chemical studies also reveal that yeast is superior to dried solubles. The total amounts of lysine and tryptophane as well as the relative amounts of these amino acids liberated by proteolytic enzymes were found to be greater in yeast than in dried solubles. Since it was found that additions of small amounts of yeast to dried solubles definitely increased growth, productions of dried solubles with a higher content of yeast would be desirable from a nutritional viewpoint.

Although the dried solubles product used in these experiments was decidedly deficient in lysine and tryptophane, it is possible that it could be used as a source of protein in rations of farm animals providing these deficiencies are corrected by other constituents of the ration.

SUMMARY

The nutritive value of distillers' dried solubles as a source of protein was evaluated by means of biological tests with rats and by chemical analyses. It was found to be inadequate as the sole source of protein and as a protein supplement because of deficiencies of lysine and tryptophane.

Since the proteins of solubles are derived from the protein of yeast and cereal grains, a study was made of the nutritive value of dried brewers' yeast and various combinations of yeast and solubles for the purpose of determining the value of yeast in the fortification of solubles. It was found that yeast was of greater nutritive value than dried solubles as the sole source of protein and effectively supplemented solubles, corn and combinations of these products.

Chemical studies revealed that solubles contain considerably less lysine and tryptophane than yeast. Furthermore, digestion experiments with proteolytic enzymes indicated that these amino acids are less available in solubles than in yeast. Since it was found that the addition of small amounts of yeast to dried solubles definitely increased its nutritive value, production of dried solubles with a higher yeast content would be desirable.

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CALCIUM METABOLISM OF PRESCHOOL CHILDREN

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Because of the high requirement for calcium for optimal skeletal development, and the relatively small amounts in all foods except milk and milk products, it is important to include in the diet foods which can be shown to favor calcium retention. Reports in the literature on the effect of orange juice on calcium retention are conflicting (Chaney and Blunt, '25; Daniels and Everson, '37; and Shepherd, Macy, Hunscher and Hummel, '40), making it seem important to study further the possible influence of this food, and certain constituents of it, on mineral retention. The data presented here are part of a larger study on the influence of changes in the ascorbic acid and citrate content of the diet on the ascorbic acid, citric acid, calcium, phosphorus, and nitrogen metabolism of eight preschool children. The effect of these supplements on ascorbic acid has been reported by Meyer and Hathaway ('44), and on citrate metabolism by Metcalf and Hathaway ('45); their effect on phosphorus and nitrogen metabolism will be reported later.

EXPERIMENTAL

Plan of the experiment. Two groups of four children each lived at the college laboratory apartment for 5 months. Subjects A, C, F, G, and H were girls, aged 55, 49, 49, 40 and 38 months, respectively, at the beginning of the study. Their respective weights at that time were 50, 38, 40, 32, and 29 pounds. Subjects B, D, and E were boys, aged 51, 44, and 55 months, and weighing 36, 35, and 34 pounds, respectively

The general plan of the complete experiment has been reported in detail by Meyer ('43), but the following facts are particularly pertinent to this part of the study. The children were maintained on a basal diet adequate in all nutrients except ascorbic acid. One teaspoonful of cod liver oil was given daily to each child. The first group of children (A, B, C, and D), were given 800 ml. of milk daily; the other group (E, F, G, and H), received only 500 ml., since it was considered that at the lower calcium level possible effects of the dietary supplements might be

more marked. Through appropriate changes in the basal diet, particularly an increase in the meat and eggs, similar levels of protein were maintained in the diets of the two groups. The calcium-phosphorus ratio was reduced slightly, from 0.9 to 0.8, on the lower intake of milk. The first 4 weeks were considered a period of personal adjustment to the new surroundings, and of metabolic adjustment to the diet. During these preliminary periods the children each received at least 1 gm. of calcium daily, the low-milk group being given an additional 0.268 gm. of calcium per day as calcium gluconate (two 1.5 gm. tablets [1] per day), in order to assure comparable calcium stores in all eight children. Somewhat smaller amounts of the basal foods were consumed by subject H throughout the study, and by subject G during the first 4 and last 5 weeks, but since milk and all supplements were given at the same level to all subjects in a group, there was little difference in the calcium intake for the different members of a group. Slight increases in the calcium content of the milk as analyzed account for the somewhat higher calcium intakes during periods 9 and 19 for the high-milk group, and 12 and 17 for the low-milk group. Supplements of crystalline ascorbic acid (100 mg.), potassium citrate (3.38 gm.) or both were given as indicated in table 1. These amounts correspond roughly to the amounts of ascorbic acid and of citrate-ion found in 200 ml. of orange juice. During the last 2 weeks the low-milk group was given orange juice in place of the crystalline supplements.

Collection and preparation of samples for analyses. The collection and preparation of urine and food samples have been described by Metcalf and Hathaway ('45). Feces were marked with carmine. The combined feces for each weekly period were digested with 1:4 hydrochloric acid according to the method of Stearns ('28–'29), made to 2000 ml. and aliquots stored for analysis.

Calcium determinations. The calcium content of the foods and excreta was determined using the following modifications of the McCrudden ('11) method. The ash from the foods and feces samples was fused with sodium carbonate according to the method of Frear and Kahlenberg ('33). To remove the supernatant from the precipitated calcium oxalate, Pyrex sintered-glass immersion tubes of fine porosity were used the second year. Hot normal sulphuric acid was then used to dissolve the calcium oxalate, and to assure its complete removal from the immersion tube.

[1] Squibb

RESULTS AND DISCUSSION

The data from this study are presented in table 1. Data for the preliminary adjustment periods have been omitted. The calcium retentions during these four periods were generally higher than those during the periods reported, but were not consistently so.

Calcium retentions. Before considering whether the various supplements were important in determining the calcium retained by these children, it seemed advisable to ascertain that the calcium retentions were normal. Average retentions of 112 ± 36 mg. to 217 ± 154 mg. have been reported for children of this age range (Macy, '42; Outhouse et al., '39; Kinsman et al., '39; Pierce et al., '40; Hawks et al., '42; and Daniels, '41). The average retentions for the eight children over the 16 weeks of the present study ranged from 76 to 156 mg. per day. The values were 131 ± 50 mg. for the four children on the high-milk intake, and 96 ± 26 mg. for those on the low-milk intake. In each group one child had values much lower than the rest, accounting for the rather large standard deviation in the values. Although these values are below some of the average values they are well within the ranges reported and it may be assumed that the calcium metabolism of the eight subjects was normal.

A summary of the calcium balances in mg./kg. body weight is given in table 2. When the data are grouped according to intake per kilogram (subjects A, E, F, and G, 39 to 50 mg./kg.; subject H, 52 to 56 mg./kg.; and subjects B, C, and D, 59 to 68 mg./kg.), the retentions are somewhat more related to the intakes: 3.2 to 6.4 mg. at the lowest level, 6.5 to 10 mg. for subject H, and 5.6 to 11 mg. for the highest level. Although the intakes for subject H are intermediate, the retentions correspond to those found for the children on the highest intake. Compared with the values for the mean daily calcium retentions reported in other studies (Macy, '42; Outhouse et al., '39) the mg./kg. values for all but subject A seem to be normal. She was overweight when she came on the study, and her retentions per kilogram are naturally lower than if her weight had been normal.

Effect of ascorbic acid supplement on calcium retention. In a review, Reid ('43) has summarized the interrelationships of calcium and ascorbic acid in various metabolic processes (growth, water balance, cell permeability, and muscle action) and has stated that a deficiency of either of these substances may affect adversely the retention or metabolism of the other. Any effect of an excess of ascorbic acid on the retention of calcium should be apparent in a comparison of the results obtained with diets I and II, and diets III and IV, table 1. The results

TABLE 1

Average daily calcium balance for four preschool children on high-milk intake.

SUBJECT		A				B				C				D			
Diet	Period	Intake	Excretion Urine	Excretion Feces	Retention	Intake	Excretion Urine	Excretion Feces	Retention	Intake	Excretion Urine	Excretion Feces	Retention	Intake	Excretion Urine	Excretion Feces	Retention
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I [1]	5	1128	..	....	...	1127	139	870	118	1128	132	874	122	1125	110	919	97
	6	1128	36	1037	55	1124	141	814	169	1127	142	890	95	1125	120	941	64
	10	1081	21	1003	57	1079	122	833	124	1081	146	881	54	1076	89	899	88
	11	1108	24	983	101	1102	130	863	109	1108	149	878	81	1101	109	786	206
	12	1145	27	993	124	1141	139	848	153	1145	149	841	154	1141	134	840	167
	Av.	1118	27	1004	84	1115	134	846	135	1118	144	873	101	1114	112	877	124
II [2]	7	1081	34	1000	48	1078	135	868	75	1081	144	809	128	1076	111	799	167
	8	1122	36	1057	28	1120	138	823	159	1121		.	...	1117	120	841	156
	9	1186	24	1050	113	1185	135	870	180	1187	150	862	175	1182	115	807	260
	13	1118	30	1019	68	1113	160	862	91	1116	142	858	116	1114	147	822	145
	14	1121	31	987	103	1114	153	841	121	1118	152	835	131	1114	..	.	...
	Av.	1126	31	1023	72	1122	144	853	125	1125	147	841	137	1121	123	817	182
III [3]	15	1105	17	1020	68	1102	100	883	119	1104	104	817	183	1103	82	858	164
	16	1127	14	967	146	1122	92	882	148	1125	104	817	203	1122	76	791	255
	17	1123	12	1020	92	1119	89	864	165	1122	97	937	88	1119	82	950	88
	Av.	1118	14	1002	102	1114	94	876	144	1117	102	857	158	1115	80	866	169
IV [4]	18	1096	8	973	116	1092	92	855	145	1095	95	786	214	1092	72	811	209
	19	1164	13	990	160	1160	103	921	137	1162	108	866	189	1160	87	911	162
	20	1137	9	1040	89	1133	77	936	120	1136	85	838	213	1135	84	935	115
	Av.	1132	10	1001	122	1128	91	904	134	1131	96	830	205	1129	81	886	162
Gen. av.		1123	22	1009	91	1119	122	865	133	1122	127	853	143	1119	103	861	156

Average daily calcium balance for four preschool children on low-milk intake.

SUBJECT		E				F				G				H			
I [1]	7	796	47	642	107	796	76	611	109	796	28	665	103	759	30	539	190
	8	808	46	662	100	776	76	611	89	776	26	682	68	773	30	626	117
	9	800	46	709	45	765	77	628	60	765	28	658	79	766	29	623	114
	Av.	801	46	671	84	779	76	617	86	779	27	668	83	766	30	596	140
II [2]	5	776	67	625	84	778	85	588	105	778	36	673	69	744	36	575	133
	6	762	52	670	40	765	81	593	91	766	34	654	78	733	36	604	93
	10	819	53	696	70	787	78	608	101	786	29	661	96	787	33	634	120
	11	811	46	672	93	779	71	606	102	766	21	633	112	768	22	633	113
	12	834	52	701	81	802	73	650	79	799	25	674	100	800	27	631	142
	Av.	800	54	673	74	782	78	609	96	779	29	659	91	766	31	615	129
III [3]	13	773	36	664	73	741	55	559	127	735	15	611	109	742	17	627	98
	14	779	..	....	..	748	60	574	114	741	18	615	108	745	13	646	86
	15	794	32	667	95	759	64	615	80	749	19	628	102	752	12	627	113
	Av.	782	34	665	84	749	60	583	107	742	17	618	106	746	14	633	99
IV [4]	16	811	37	716	58	778	70	664	44	736	16	643	77	760	14	669	77
	17	850	34	727	89	818	58	615	145	775	17	634	124	798	14	678	106
	18	792	26	717	49	753	53	593	107	714	19	589	106	739	17	624	98
	Av.	818	32	720	65	783	60	624	99	742	17	622	102	766	15	657	94
V [5]	19	806	46	685	75	771	71	627	73	730	25	587	118	741	22	623	96
	20	825	49	693	83	791	82	574	135	738	23	657	58	756	22	621	113
	Av.	815	47	689	79	781	76	600	104	734	24	622	88	748	22	622	104
Gen. av.		804	45	683	76	775	71	607	98	759	24	642	94	760	23	624	113

[1] Basal diet only.
[2] Basal diet plus ascorbic acid.
[3] Basal diet plus potassium citrate.
[4] Basal diet plus ascorbic acid and potassium citrate.
[5] Basal diet plus orange juice.

TABLE 2

Summary of calcium balances for eight preschool children.

		INTAKE	EXCRETION		RETENTION	
			Urine	Feces		% of intake
		mg./kg.	*mg./kg.*	*mg./kg.*	*mg./kg.*	
Subject A						
Diet	I [1]	49	1.2	44	3.7	8
	II [2]	50	1.4	45	3.2	6
	III [3]	49	0.6	44	4.5	9
	IV [4]	49	0.4	44	5.3	11
Subject B						
Diet	I [1]	67	8.1	51	8.1	12
	II [2]	68	8.7	51	7.5	11
	III [3]	65	5.5	51	8.4	13
	IV [4]	65	5.3	52	7.7	12
Subject C						
Diet	I [1]	62	8.0	49	5.6	9
	II [2]	62	8.1	46	7.5	12
	III [3]	59	5.4	45	8.4	14
	IV [4]	60	5.1	44	10.8	18
Subject D						
Diet	I [1]	67	6.7	53	7.4	11
	II [2]	66	7.2	48	10.7	16
	III [3]	63	4.5	49	9.5	15
	IV [4]	63	4.5	49	9.0	14
Subject E						
Diet	I [1]	50	2.9	42	5.2	10
	II [2]	49	3.3	42	4.6	9
	III [3]	47	2.0	40	5.1	11
	IV [4]	48	1.9	42	3.8	8
	V [5]	47	2.7	40	4.6	10
Subject F						
Diet	I [1]	42	4.1	33	4.6	11
	II [2]	42	4.2	33	5.1	12
	III [3]	39	3.1	31	5.6	14
	IV [4]	40	3.1	32	5.1	13
	V [5]	39	3.8	30	5.3	13
Subject G						
Diet	I [1]	49	1.7	42	5.3	11
	II [2]	49	1.8	41	5.7	12
	III [3]	45	1.0	37	6.4	14
	IV [4]	45	1.0	38	6.2	14
	V [5]	44	1.5	38	5.3	12
Subject H						
Diet	I [1]	56	2.2	43	10.1	18
	II [2]	56	2.2	45	8.7	16
	III [3]	53	1.0	45	7.0	13
	IV [4]	53	1.1	46	6.5	12
	V [5]	52	1.5	43	7.2	14

[1] Basal diet only.
[2] Basal diet plus ascorbic acid.
[3] Basal diet plus potassium citrate.
[4] Basal diet plus ascorbic acid and potassium citrate.
[5] Basal diet plus orange juice.

were inconsistent: the calcium retention was increased for subjects D, F, and G when ascorbic acid was added to the basal diet, but not when it was added to the basal diet plus potassium citrate, but the reverse was true for subject A. The retentions for subjects B, E, and H were less when ascorbic acid was added to both of the diets, but were more for subject C in both cases. Comparison of retention values for all the periods when ascorbic acid was added (diets II and IV) with those when it was omitted (diets I and III) showed that the differences were not statistically significant for any child. These results check those of Daniels and Everson ('37) rather than those of Shepherd and associates ('40), i.e., ascorbic acid in excess of that in the basal diet did not significantly increase the retention of calcium in these preschool children.

Effect of potassium citrate supplement on calcium retention. Studies by Shohl ('37) and Hamilton and Dewar ('37) have shown that citric acid and its salts have antirachitogenic action in the rat. The work of Hathaway and Meyer ('39) confirmed the effectiveness of citrates in improving calcification and indicated that potassium citrate was more effective than the sodium salt or a mixture of citric acid and its sodium salt. It seemed possible, therefore, that the citrates present in orange juice might have been a factor in the increased calcium retention found in some human subjects when orange juice was added to the diet. Consequently 3.38 gm. of potassium citrate were added to the diets of these 8 children. This is the amount calculated to contain the same citrate-ion content as 200 mg. of orange juice. The results are given under diets III and IV in table 1.

In every period during which potassium citrate was given (diets III and IV) the urinary calcium excretion was less than when it was omitted. The fecal excretions during these periods, and consequently the retentions of calcium, were irregular. The retentions for subject C were significantly higher when the salt was added, those for subject H were significantly higher without the salt, and the differences were not statistically significant for the other six children. It may be concluded that although potassium citrate ingestion significantly reduces the urinary excretion of calcium, undetermined factors affecting its absorption from the intestine may overbalance this decreased urinary excretion so that the retention may be little affected.

Effect of orange juice supplement on calcium retention. An amount of orange juice containing 110 mg. of ascorbic acid (averaging 218 ml.) was added to the basal diet of one group of the children during the last two periods, and the results are recorded under diet V, part 2 of table 1. The average retentions of three of the four children were

slightly higher during these two periods than in the preceding three periods on potassium citrate plus ascorbic acid, but not significantly so. The urinary excretions were higher in all cases than on the potassium citrate supplement, but the fecal excretions were sufficiently lower to result in slightly higher retentions. The differences in response for the periods on orange juice supplement make questionable the significance of these increases in retention.

Again these results agree with those of Daniels and Everson ('39) rather than with those of Shepherd and associates ('40). In the studies of Chaney and Blunt ('25) and those of Ludwig and Schuck ('41) on older subjects, only short preliminary periods and short experimental periods were used, so that they are not comparable with the present study.

Fecal excretion and intestinal absorption of calcium. The average daily fecal excretions of calcium ranged from 853 to 1009 mg. on the high-milk intake, and from 607 to 683 on the low-milk intake. Greater variation was found from period to period, and from child to child than was found for the urinary excretions of calcium. This was due, in part at least, to the difficulty in separation of feces between periods, and to the much higher concentration of calcium in fecal material.

Marked differences in rate of defecation and nature of the stools was noted among the eight children: subjects D and F were frequently constipated, and subjects E and H often had unformed stools. Fecal calcium excretion, however, did not show much relationship to rate of defecation: e.g., subject A with normal defecation, excreted the most calcium in the feces, and subject H, with rapid defecation averaged less fecal calcium than subject G.

Since there is extensive evidence that calcium is not actively excreted into the intestinal tract, the intake minus the fecal output may be considered to be the calcium absorbed. On this basis, the average daily absorptions were 113, 255, 269 and 258 mg. or 10 to 24% of the intake for the high-milk group, and 112, 169, 118 and 136 mg. or 14 to 22% of the intake for the low-milk group. The absorption for A was abnormally low in comparison with that of the other three subjects in her group, and her retention was of necessity much below theirs. Subjects C and F, however, who apparently absorbed more calcium than the other subjects in their groups, did not have higher retention values. Since the urinary output of subjects A, G, and H averaged only 22 to 24 mg. per day, it may be that absorption was one factor affecting the amount which they retained. For the other five subjects, however, this would not appear to be the main controlling mechanism.

Urinary excretion of calcium. The urinary excretions of calcium were not uniform from child to child, but were relatively consistent for each child on a given supplement. They were little affected by additions of ascorbic acid to either the basal diet or to the basal diet plus potassium citrate, but the addition of potassium citrate (diets III and IV) resulted in significantly lower urinary calcium excretions in all eight children. This decrease averaged from 10 to 53 mg. for the different subjects, but was relatively consistent and characteristic for each child. These results lend support to the suggestion that urinary calcium excretion may be related to acid-base balance. Addition of potassium citrate to the diet increased the average urinary pH by 0.9 to 1.4 units, from averages of 5.5 to 5.9, to from 6.7 to 6.9. With an increased urinary pH, the urinary calcium excretion was reduced. When orange juice was added to the diet of the low-milk group, the urinary excretion of calcium was about the same as on the basal diet for E and F, but somewhat lower for G and H. It was higher than on the potassium citrate supplement in all subjects. The addition of orange juice increased the basal pH values by about 0.3 units for E and F, and by about 0.6 units for G and H. These results also lend possible support to a relationship between urinary calcium excretion and acid-base balance.

Knapp and Stearns ('44) state that urinary calcium bears an important relationship to calcium intake and body weight. According to their formulas for calculation of the normal percentage of calcium intake which is excreted in the urine, the children from the high-milk group should have values between 1.0 and 16%, and those from the low-milk group between 1.2 and 19%. The percentage values found were as follows: A, 0.8 to 3.2; B, 6.8 to 14; C, 7.7 to 14; D, 6.6 to 13; E, 3.2 to 8.6; F, 7.4 to 11; G, 2.2 to 4.6; and H, 1.6 to 4.9. Thus subjects A, G, and H eliminated amounts of calcium definitely in the lower normal range; subjects B, C, and D definitely in the higher normal range; and only subjects E and F in the middle range, regardless of supplement. Knapp and Stearns recognized that the major factor regulating urinary excretion is endogenous, and believe that it is probably related to endocrine balance. Parathyroid and thyroid activity may be involved, but the exact relationship has not been determined.

SUMMARY AND CONCLUSIONS

Calcium metabolism has been studied in eight preschool children, four on average calcium intake of 1122 mg., and four on an intake of 775 mg. The basal diet was supplemented with ascorbic acid, potassium citrate, and orange juice as indicated. The results were as follows:

1. Calcium retentions during 16 weeks averaged 131 ± 50 mg. on the higher level of intake, and 96 ± 26 mg. on the lower level.

2. The addition of 100 mg. of ascorbic acid to a diet containing 23 to 25 mg. of the vitamin did not significantly increase the retention of calcium.

3. The addition of 3.38 gm. of potassium citrate lowered the urinary excretion of calcium in all eight subjects but did not significantly alter the calcium retention.

4. On the substitution of orange juice for crystalline ascorbic acid and potassium citrate supplements, the retentions of calcium were not significantly altered.

5. In at least five of the eight subjects the absorption of calcium did not appear to be the main factor regulating its retention.

6. In these eight children factors other than diet alone regulated calcium retention during the 16 weeks of observation.

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THE PROTEIN REQUIREMENTS OF ADULT HUMAN SUBJECTS IN TERMS OF THE PROTEIN CONTAINED IN INDIVIDUAL FOODS AND FOOD COMBINATIONS [1]

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ONE FIGURE

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Most of what we know, or think we know, about human nutrition has been obtained from experimental observations upon laboratory animals. Nutrition experiments upon human subjects are difficult to carry out, because of the inconvenience to the subjects of the conditions that must be imposed for the most effective interpretation of the results to be secured, not the least of which is the great divergence between the experimental diets that must be used and the diets to which the subjects have been habituated. This is particularly true of studies in protein nutrition in which low-protein diets must be tolerated for many days. The usual diets consumed by adults contain two or three (or more) times the amount of protein required for nitrogen equilibrium.

Determinations of protein utilization in digestion and in metabolism upon growing rats are readily made and the measurements obtained may be accurate from the standpoint of reproducibility. However, there is always some degree of doubt as to their applicability to the human, and until more information on the value of the rat as a "pilot" animal in human nutrition is at hand, this doubt will remain. Meantime there is a real need for protein studies on human subjects.

A number of experimental methods have been employed in studies of protein nutrition in adult men and women: the method of Thomas ('09) involving the computation of biological values in the original meaning of the term; the method of Murlin and associates (Sumner, Pierce and

[1] The authors gratefully acknowledge the assistance in carrying out this experiment of funds donated by the Graduate School of the University of Illinois.

[2] The data reported in this paper were taken from a thesis presented by Mildred Bricker to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy.

Murlin, '38) in which the Thomas method is modified by use of an egg protein diet as a standard rather than a nitrogen-free diet; and the method of Sherman (Sherman, Winters and Phillips, '19), in which different sources of protein are compared on the basis of the balances of nitrogen secured on comparable, and low, intakes of nitrogen. None of these methods is ideal in experimentation with adult human subjects, either because of the difficulty in tolerating the experimental diets, the purely comparative nature of the results secured, or the impossibility of computing a protein requirement for nitrogen equilibrium.

However, if the relationship in adult nutrition between the nitrogen intake and the nitrogen balance (or ouput) is rectilinear below the point of equilibrium, and even somewhat above it, a more practicable method is at hand for studying with human subjects the utilization of dietary nitrogen and the requirements for equilibrium of nitrogen from different dietary sources. Martin and Robison ('22) established the rectilinear character of the relationship for human adults between the nitrogen intake and output when the dietary nitrogen was supplied by whole wheat flour, and less certainly when it was furnished by milk. In their selection from the literature of metabolism data for the determination of the protein requirement for maintenance in man, Leitch and Duckworth ('37) were able to describe satisfactorily the relationship between nitrogen intake and nitrogen output by straight lines. Melnick and Cowgill ('37) report remarkably close rectilinear relationships for adult dogs between nitrogen intake and nitrogen balance for dietary proteins differing widely in biological value, i.e., lactalbumin, serum protein, casein and gliadin. The intercept of the straight lines describing this relationship on the abscissa representing nitrogen equilibrium, gives the amount of nitrogen, of the species present in the diet, required for equilibrium. The intercept on the ordinate erected at zero nitrogen intake, gives the minimum endogenous output of nitrogen in the urine plus the metabolic nitrogen in the feces. This method was used by Harris and Mitchell ('41) in estimating the requirements of nitrogen in the form of urea and of casein for the maintenance of nitrogen equilibrium in adult sheep. It was used in the investigation on human subjects to be reported in this paper.

EXPERIMENTAL PROCEDURES

The subjects of the experiment were nine young women attending the University of Illinois. Their description with reference to age, weight, height and basal metabolism is given in table 1. The basal metabolism was determined by the gasometer method. Two or more

measurements were made upon each subject, depending upon the agreement secured between the first two. The nitrogen metabolism data for different subjects were pooled together by expressing them in milligrams per basal calorie in accordance with the observations of Smuts ('35) confirming those of Terroine that the minimum endogenous catabolism of nitrogen, to which the maintenance requirement of protein is due, varies with the basal metabolism.

The experimental diets were planned to contain as high a proportion of the total nitrogen in the test food as possible. The proportion realized was generally 95% or higher, though in a few cases it ranged from 91

TABLE 1

Description of the subjects of the experiments.

SUBJECTS		AGE	WEIGHT	HEIGHT	SURFACE AREA [1]	BASAL METABOLISM	
No.	Initials					per hr. per mm.^{2}	per day
		years	*kg.*	*cm.*	mm.^{2}	*cal.*	*cal.*
1	B.M.	20	54.12	167.0	1.60	32.60	1252
2	R.L.	20	61.98	167 6	1.70	31.67	1292
3	M.B.	23	58.87	158.4	1.61	35.66	1378
4	H.M.	21	56.45	172.7	1.67	31.89	1278
5	M.W.	19	54.68	159.7	1.56	33.09	1239
6	J.B.	19	57.90	159.4	1.59	32.65	1246
7	C.T.	20	61.20	164.5	1.72	34.62	1429
8	C.M.	20	75.15	167.3	1.85	31.19	1385
9	L.T.	22	55.60	166.4	1.61	31.11	1202

[1] Estimated by the height-weight formula of DuBois and DuBois ('16).

to 94%. All diets were supplemented with minerals (Ca, P, Na, Cl, Mg, K, Fe, Mn, Cu, I and S) and with the following amounts of vitamins per subject daily: 3 mg. of thiamine hydrochloride, 3 mg. of riboflavin, 15 mg. of nicotinamide, 1.5 mg. of pyridoxine and 2 mg. of calcium pantothenate. Each subject received daily 2 drops of haliver oil and 20 drops of solvent-extracted wheat germ oil.

The basal diet furnished from 0.14 to 0.23 gm. of nitrogen daily and consisted of sugar-cornstarch-lard cookies, supplemented with sucrose, lactose, fondant, jelly, butterfat, lemon juice, apple sauce, lettuce and French dressing. Water and table salt were allowed ad libitum. The test foods replaced isocaloric amounts of some of the nitrogen-free constituents of the basal diet at varying nitrogen levels. The diets were all consumed at caloric levels just adequate to maintain a constant body weight. The estimated physiological fuel value required ranged from 1.6 to 2.4 times the basal energy expenditure, averaging about 2.0 times. No attempt was made to standardize the proportion of food calories de-

rived from fat, which varied rather widely in individual periods but for the different foods averaged from 26 to 42%. This circumstance did not appear to contribute to the variability of the data secured. Farr ('39) has shown that a five-fold variation in the fat intake of two children with the nephrotic syndrome on isocaloric diets had no appreciable effect on the nitrogen balance.

The protein foods tested consisted of soy flour,[3] patent white flour,[4] milk,[5] a soy-white flour combination containing 13% of the former, supplying about 36% of the nitrogen of the mixture, and a typical mixture of foods modified from list 1 proposed by the Food and Nutrition Board of the National Research Council in Circular no. 115 issued in January, 1943. The latter mixture was consumed in three meals according to the following menu (all quantities in grams): Breakfast: oranges 120, oatmeal 27, egg 43, milk 229; luncheon: tomato soup 240, lettuce 60, peaches 120, soy wheat biscuits 48, milk 200; dinner: ground beef 65, beef suet 10, potatoes 240, carrots 120, cabbage 80, pineapple 24, soy wheat biscuit 48. For the purpose of testing the protein value of this mixture of foods, they were fed in three periods to the subjects participating in the tests in amounts equal to one-half, three-eighths and one-fourth of the quantities given above. The individual foods were also tested at different levels. All test foods, in this combination and among the individual foods, were cooked in some appropriate manner if they are commonly prepared by cooking. The milk was pasteurized. The oranges, lettuce, carrots and cabbage were served raw, and the peaches, pineapple and tomato soup were canned. The experimental meals were prepared and served in the Nutrition Laboratories of the Department of Home Economics. All test foods were analyzed for nitrogen, moisture, and ether extract by the approved methods of the A.O.A.C., and their heats of combustion (gross energy) were determined by the Parr oxygen-bomb calorimeter. The constituents of the basal diet containing appreciable amounts of nitrogen were also analyzed for this element, but their contents of water, fat and carbohydrate were estimated from tables of food composition (Chatfield and Adams, '40).

The test periods were continued until the nitrogen output in the urine, determined daily, showed no progressive change and was reasonably constant for 3 or more days. The average length of the periods was

[3] Staley's Stoy flour, containing 7.67% nitrogen.

[4] Pillsbury's enriched white patent flour, containing 2.05% nitrogen.

[5] Four per cent homogenized, pasteurized milk from the University creamery, containing an average of 0.553% nitrogen.

7.6 days, though there was considerable variation depending mainly on the type of diet consumed in the prior period.

The average daily output of nitrogen in the urine for the last 3 or 4 days of the period, or sometimes longer up to 9 days, was used in computing the nitrogen balance characteristic of the source and level of nitrogen consumed. The feces were collected for periods of 3 to 5 days and were marked off by either Fe_2O_3 or Cr_2O_3, given in 0.3 to 0.5 gm. doses. They were preserved by refrigeration and then homogenized with dilute acetic acid in a Waring Blendor.

Either four or five subjects participated in the study of each test food and for each test food there were from 7 to 13 experimental periods, making a total of 48 periods for all foods. In addition, two of the Subjects, nos. 3 and 4, completed tests on the low-nitrogen basal diet.

THE EXPERIMENTAL RESULTS

The most significant information afforded by these experiments concerns the relationship between the nitrogen intake and the nitrogen balance for the different sources of dietary nitrogen. By expressing each of these variables in milligrams per basal calorie, it is possible to pool the data for all subjects regardless of body size (Smuts, '35). This has been done in the respective graphs of figure 1. All of the individual data are plotted in these graphs, the small number beside each circled point referring to the subject supplying the measurement. The two experimental periods on the basal low-nitrogen diet for Subjects 3 and 4 are included in each graph.

The rectilinearity of the relationship between nitrogen intake and nitrogen balance for the same dietary source of nitrogen seems obvious from all charts, and is particularly evident from those for milk, white flour, and the combination of soy and white flour. Hence, equations representing straight lines of the type $Y = a + bX$ were fitted to each of the five sets of data by the method of least squares. In these equations, Y represents the nitrogen balance in milligrams per basal calorie; X represents the nitrogen intake expressed in the same terms; a represents the intercept of the line on the Y axis, or the total nitrogen loss in milligrams per basal calorie when the nitrogen intake is zero; and b defines the slope of the line, or the change in nitrogen balance in milligrams per milligram of change in nitrogen intake.

The a values in the five equations range from 1.719 to 1.924, and average 1.830 mg. per basal calorie. This value represents the total loss of nitrogen from the body on a nitrogen-free diet, expressed in milligrams per basal calorie. In the two periods with Subjects 3 and 4 subsisting

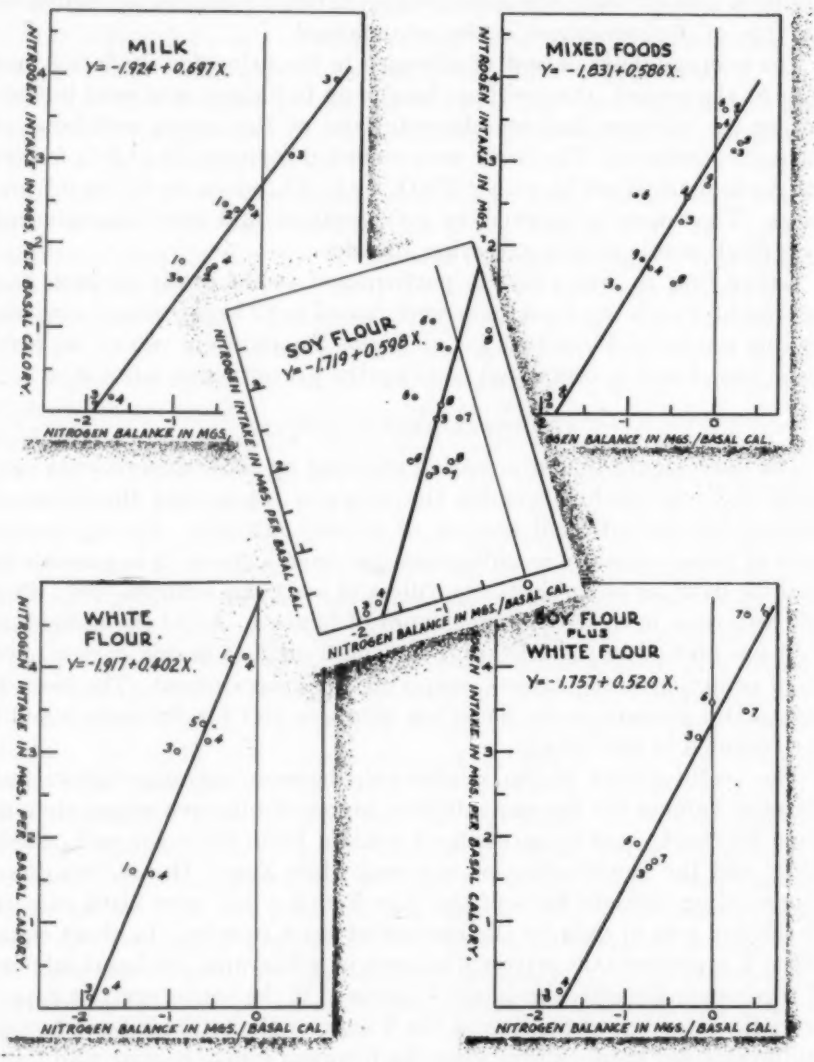
MILK
Y= -1.924 + 0.697 X.
MIXED FOODS
Y= -1.831 + 0.586 X.
SOY FLOUR
Y= -1.719 + 0.598 X.
WHITE FLOUR
Y= -1.917 + 0.402 X.
SOY FLOUR PLUS WHITE FLOUR
Y= -1.757 + 0.520 X.
NITROGEN INTAKE IN MGS. PER BASAL CALORY.
NITROGEN BALANCE IN MGS./BASAL CAL.

Fig. 1 The relationship between nitrogen intake and nitrogen balance on different diets. The Y = — a + bX equations define the relationship for the various foods, the *a* value representing the nitrogen excretion at *O* intake and the *b* value the slope of the line, or the product of the biological value and the true digestibility of the nitrogen. The numbers 1–9 adjacent to the circled points refer to the subjects yielding the data from which the calculated curves were drawn.

on a practically nitrogen-free diet, the fecal (metabolic) nitrogen amounted to 0.410 and 0.401 mg. per basal calorie, an average of 0.405. If this value is deducted from 1.830, the difference, 1.425, is the average minimum endogenous urinary nitrogen in milligrams per basal calorie.[6]

The *b* values in the five equations define the slope of the lines describing the respective relationships between the nitrogen intake and the nitrogen balance, both expressed in milligrams per basal calorie. Each value is the product of the coefficient of true digestibility computed in the usual manner and the biological value in the Thomas ('09) sense, each expressed as a decimal rather than as a percentage.[7] The *b* values with their standard errors[8] are as follows: for milk nitrogen, 0.697 ± 0.042; for the nitrogen of white flour, 0.402 ± 0.027; for the nitrogen of soy flour, 0.598 ± 0.086; for the nitrogen of the soy-white flour combination, 0.520 ± 0.046; and for the nitrogen of the mixed food combination, 0.586 ± 0.059. Thus, the average over-all nutritive quality of the different sources of dietary nitrogen ranges as follows in the order of decreasing average quality: milk, soy flour, mixed foods, soy flour plus white flour, and white flour.

The statistical significance of the differences in *b* values have also been computed with the results summarized in table 2. If the probabili-

[6] Smuts ('35) found an average of 2 mg. of endogenous urinary nitrogen per basal calorie for a series of mice, rats, guinea pigs, rabbits and pigs, mainly adult males, and from the meager information available concluded that approximately the same ratio applied to men. However, the possibility of a sex difference in this respect was not considered. Palmer, Means and Gamble ('14) observed a marked sex difference in the ratio of urinary creatinine excretion to basal metabolism, such that for men 1.02 mg. of creatinine were excreted per basal calorie, while for women the ratio was only 0.8 as much. If it may be assumed that this sex difference applies to the total output of minimal endogenous urinary nitrogen, the Smuts' ratio of 2 mg. per basal calorie for men would correspond to a ratio of 1.6 mg. per basal calorie for women. This value is not greatly different from that of 1.425 estimated for the women subjects of this experiment.

[7] The subscripts to N in the following equations relate to the different factors entering into a nitrogen metabolism study, N standing for nitrogen in all cases: b = balance, i = intake, f = fecal, u = urinary, m = metabolic fecal, and e = minimum endogenous urinary. In these terms the regression line relating nitrogen balance to nitrogen intake becomes — $N_b = -a + bN_i$, in which $a = N_m + N_e$ and $N_b = N_i - N_f - N_u$.

Substituting and rearranging — $b = \dfrac{N_i - (N_f + N_m) - (N_u - N_e)}{N_i}$.

The coefficient of true digestibility of nitrogen, in these terms, would be equal to $\dfrac{N_i - (N_f - N_m)}{N_i}$, and dividing this into b —

$$b \cdot \frac{N_i}{N_i - (N_f - N_m)} = \frac{N_i - (N_f - N_m) - (N_u - N_e)}{N_i - (N_f - N_m)} = \text{the biological value.}$$

[8] The standard errors were computed according to the method of Rider ('39, pp. 93, 94), as well as the statistical significance of the differences in *b* values.

ties (last column of this table) that a random combination of the uncontrolled factors in the experiment would by themselves produce differences as great or greater than those observed are as small or smaller than 0.030, they may reasonably be neglected. In such cases, it may be concluded that the differences are real, being due to actual differences in the over-all nutritive qualities of the sources of dietary nitrogen compared. On the basis of this reasoning, it may be concluded that milk nitrogen is definitely superior to the nitrogen of white flour and of soy flour plus white flour in the combination used. Patent white flour is definitely inferior to all other sources of dietary nitrogen tested. No difference in over-all nutritive quality has been demonstrated in any of the other comparisons, although the data suggest the superiority of milk nitrogen over that of the mixed foods. However, since milk nitro-

TABLE 2

Statistical significance of differences in b values.

FOODS COMPARED	DIFFERENCE IN b VALUES	n	t	P
Milk and white flour	.295	16	5.931	$<.005$
Milk and soy flour	.099	20	1.036	ca. .15
Milk and soy + white flour	.177	17	2.705	.008
Milk and mixed foods	.111	22	1.417	.088
White flour and soy flour	.196	18	2.200	.022
White flour and soy + white flour	.118	15	2.077	.029
White flour and mixed foods	.184	20	2.618	.0086
Soy flour and soy + white flour	.078	19	.818	$>.15$
Soy flour and mixed foods	.012	21	.114	$>.15$
Mixed foods and soy + white flour	.066	21	.881	$>.15$

gen is definitely better than the nitrogen in the combination of soy flour and white flour, while neither soy flour nitrogen nor the nitrogen of the mixed foods has been so characterized, it would seem reasonable to interpret the entire picture as establishing the following three classes of dietary nitrogen:

(1) Milk; (2) soy flour, soy flour and white flour, mixed foods; and (3) white flour. This statement does not imply that real differences do not exist among the foods of Class 2. The variability of the data was such that differences have not been established statistically.

If the nitrogen of soy flour and that of white flour exerted no mutual supplementary effects when combined in the proportion of 36 parts of the former to 64 parts of the latter, then the b value of the combination would be 0.473. However, the statistical significance of the difference between this value and that observed for this combination, i.e., 0.520,

cannot be established, mainly because of the large standard error of the *b* value of soy flour. The data suggest the existence of a supplementary relation.

The fecal nitrogen in these experiments seemed to be largely of body, rather than of food, origin. When expressed per gram of dry matter consumed, there was no demonstrable correlation with the nitrogen intake, that is, with the nitrogen content of the dry matter consumed. Furthermore, the average amounts of fecal nitrogen in milligrams per gram of dry matter consumed varied within a small range for all test diets, the averages with their standard errors being as follows: milk, 1.18 ± 0.21; white flour, 1.29 ± 0.13; soy flour, 1.42 ± 0.17; mixture of white and soy flour, 1.37 ± 0.12; and mixed foods, 1.78 ± 0.13. In the two periods on the low-nitrogen basal diet, the fecal nitrogen amounted to 0.91 mg. per gram of dry matter consumed for Subject 4, and 1.14 mg. for Subject 3.

The average coefficients of apparent digestibility of the nitrogen in the different foods and food combinations tested were: 77.2 for milk, 81.6 for white flour, 70.3 for soy flour, 77.9 for the combination of soy and white flour, and 75.8 for the mixed foods.

The true digestibility of the nitrogen of the foods and combinations tested can be computed by assessing the metabolic nitrogen in the feces at 1 mg. per gram of dry matter consumed, this value being the average of the two values obtained with Subjects 3 and 4 on the basal low-nitrogen diet, 0.908 and 1.140. The true digestibilities thus computed are as follows: 94 for milk, 97 for white flour, 92 for soy flour, 94 for the combination of soy and white flour, and 90 for the mixed foods. Because of the large amounts of foods in all of the experimental diets contributing practically no nitrogen to the intake, but inducing an excretion of nitrogen in the feces in proportion to the amounts of dry matter they contain, these coefficients of true digestibility are of greater significance than the coefficients of apparent digestibility given in the preceding paragraph.

The amounts of nitrogen required for equilibrium may be computed from the equations relating intake and balance of nitrogen for the various test foods by equating the balance (Y) to zero and solving for X. The values thus obtained are summarized in table 3 for the various foods and food combinations tested, together with other information pertinent to the nutritive evaluation of their protein components.

The values given in the last two columns of this table are the most significant results secured in these experiments, defining as they do the amounts of nitrogen and of conventional protein required for mainte-

nance (as ordinarily determined) in the adult organism. The nitrogen requirement for the most general use is expressed in milligrams per basal calorie. This method of expression takes account of differences in body size more effectively than the customary method relating requirement to body weight.

While on the white flour diet providing 6.819 gm. of nitrogen daily, Subject 3 excreted 6.813 gm. of nitrogen daily in the urine during a 5-day period and was in negative nitrogen balance to the extent of 0.872 gm. daily. For the next 5 days she consumed essentially the same diet plus a daily supplement of lysine, providing 0.263 gm. of nitrogen.

TABLE 3

The nutritive value of the nitrogen components of the foods and food combinations tested.

TEST FOODS	TRUE DIGESTIBILITY PCT.	BIOLOGICAL VALUE [2] PCT.	REQUIRED FOR EQUILIBRIUM	PROTEIN (N × 6.25) REQUIRED PER DAY [3]
			mg. per basal cal.	*gm.*
Milk	94.0	74	2.760	22.4
White flour	96.9	41	4.763	38.7
Soy flour	91.9	65	2.876	23.4
Soy-white flour [1]	94.2	55	3.380	27.5
Mixed foods	89.6	65	3.124	25.4

[1] Thirty-six parts of soy bean protein to 64 parts of white flour protein.

[2] Computed from the respective *b* value in the equation expressing the relation between N intake and N balance, by dividing by the true digestibility expressed as a decimal.

[3] For a basal metabolism of 1300 cal. daily, the average for the nine subjects of this experiment.

Immediately the urinary nitrogen dropped by almost 0.9 gm. daily and the subject went into positive balance. This result confirms the experience of Rose ('44) and of Albanese et al. ('41) that the adult human requires lysine, and brings out a difference in this respect between adult human and adult rodent nutrition (Burroughs, Burroughs and Mitchell, '40).

DISCUSSION

The biological values for the nitrogen in the foods and food combinations tested, given in table 3, may be compared with the few others reported in the literature, although there is no high degree of agreement. For milk protein, Sumner and Murlin ('38) report a biological value of 62, Lintzel and Bertram ('38) one of 43, and Martin and Robison ('22) one of 51, for adult humans, compared with our value of 74. Wagner ('23) obtained a value for milk proteins of 60 with a group of children with latent hilus gland tuberculosis. The differences are largely unex-

plainable, although in some cases, it may be questioned whether the true minimal endogenous urinary nitrogen was ever attained, or whether the caloric intake was adequate (Lintzel and Bertram, '38). In the present experiments the biological values given relate to a definite caloric intake, that required for energy equilibrium. A larger, or a smaller, caloric intake may be expected to change the picture by sparing dietary protein to a greater or lesser extent, as Neumann ('19) and Cuthbertson and Munro ('37, '39) have shown. It would seem that the most significant biological values for the adult human would relate to energy intakes just sufficient to maintain the body weight.

For soy proteins, an average biological value of 65 was reported for soybean curd by Cheng, Li and Lan ('41), comparing favorably with the value of 65 for soy flour given in table 3. The high value of 81 for soybean protein reported by Murlin et al. ('44) may probably be explained by the short low-nitrogen period of 3 days, during which time the minimal level of urinary nitrogen may not have been established.

The nitrogen requirements for equilibrium given in table 3 may be computed in terms of conventional protein ($N \times 6.25$) for the conventional body weight of 70 kilograms. Such a person would have a surface area of about 1.8 square meters and a basal metabolism of approximately 1650 cal. per day. Hence, his daily requirement of protein from the sources tested would be: from milk, 28.5 gm.; from white flour 49.1 gm.; from soy flour, 29.7 gm.; from soy flour 13 parts and white flour 87 parts, 34.9 gm.; and from the mixed diet, 32.2 gm. If the ratio of minimum endogenous urinary nitrogen to basal calories is less in women than in men, as the results of Palmer, Means and Gamble ('14) may suggest, the above values should be increased by about 18%.[9]

The above estimates of protein needs in adult life assume that the nitrogen output from the body is composed solely of the urinary and fecal output, and that the protein requirements of adulthood are completely covered when the body is in nitrogen equilibrium. Holt ('44) has pointed out certain possible errors in this latter assumption, but the bases of his arguments need either confirmation or experimental proof. In any case their applicability to the situation under discussion is doubtful. However, it is undoubtedly true that throughout adult life some tissues are continually growing. Hrdlička ('36) has called attention to this fact with reference to stature, various head and face dimensions, the chest, hands and feet. But aside from these growth changes in body size and dimension, it is known that certain tissues of the body, such as the epidermis and epidermal structures, grow continuously. All

[9] Assuming no sex difference in the ratio of fecal metabolic nitrogen to dry matter consumed.

these growth changes involve protein, so that the adult, to maintain the body in its normal and proper nutritive status, must receive more dietary protein than that required merely for equilibrium. What this excess should be has not been assessed insofar as the authors are aware.

Grindley ('12) has reported continuous nitrogen balance studies carried out over a period of 220 days on 23 adult men from 18 to 31 years of age. They were consuming an adequate diet, containing 80 to 85 gm. of protein daily, and body weight variations were slight. The daily nitrogen balance averaged 1.38 gm., with a coefficient of variation of 22.3. The balance seemed to be appreciably correlated with body surface ($r = +0.480$), and per square meter averaged 0.77 gm., with a coefficient of variation of 19.3. This apparent storage of nitrogen by adult men can be broken down into two factors; one, a failure to measure all of the nitrogen output from the body, specifically that from the skin; and, two, a true retention of nitrogen in the growth of hair, epidermal tissues and structures, and in the changes in size and form of the body characteristic of the later phases of growth.

In unpublished experiments, Mitchell and Hamilton have measured the dermal losses of nitrogen in four adult men while at comparative rest in an air-conditioned room. Under comfortable, non-sweating conditions this loss averaged 0.40 gm. daily; under profuse sweating conditions it averaged 3.74 gm. Applying the former figure to the average positive balance of nitrogen observed in Grindley's subjects, it would appear that approximately 1 gm. of nitrogen a day (1.38 — 0.40) was actually stored in the bodies of these adult men.

An estimate of the total requirement of nitrogen by the women subjects of this experiment on the basis of the information considered above may be made by computing, from the equations relating nitrogen intake to nitrogen balance for each of the test foods, that intake of nitrogen required to induce a storage of 0.77 gm. of nitrogen per square meter of body surface. The average surface area of the subjects in this study was 1.66 $mm.^2$ and their average basal metabolism was 1300 cal. per day. Hence, the above nitrogen storage, in milligrams per basal calorie, is $(0.77 \times 1.66) \div 1300 = 0.983$ mg. The amounts of protein of the various species tested required for maintenance and "adult growth" are given in table 4.

The requirements here computed are averages relating to a nutritional status characteristic of the subjects of this experiment. For adult women in general, such requirements in all probability exhibit considerable variation. Individuals may be able to adapt themselves to lower planes of protein nutrition (Mitchell, '44), by diminishing the

inevitable wastage of nitrogen in the endogenous metabolism, or by making better use of the protein supply available through the protein-sparing action of a more abundant intake of carbohydrates.

The differences in requirement depending upon the source of the dietary protein are great. It is interesting to note that the estimated protein requirement on a mixed diet is among the lowest of those secured.

TABLE 4

The amounts of nitrogen and of conventional protein required for true maintenance and "adult growth".

SOURCE OF NITROGEN	NITROGEN REQUIRED PER BASAL CAL.[1]	PROTEIN (N × 6.25) REQUIREMENT	
		Average for the subjects of this experiment	Per 70 kg. body weight
	mg.	*gm. per day*	*gm. per day*
Milk	4.171	33.9	43.0
White flour	7.214	58.6	74.4
Soy flour	4.518	36.7	46.6
Soy-white flour combination	5.269	42.8	54.3
Mixed foods	4.802	39.0	49.5

[1] These figures were obtained by substituting + 0.983 mg. N for the Y in the respective $Y = a + bX$ equations, and solving for X.

SUMMARY AND CONCLUSIONS

Fifty nitrogen balance periods were carried out upon nine women subjects to determine the requirements for protein during adult life for different types of dietary protein. The protein foods tested were milk, soy flour, white flour, a combination of soy flour and white flour containing 13% of the former, and a well balanced mixture of protein foods. All test foods were cooked in some appropriate manner if they are commonly prepared by cooking. The test foods supplied 91% or more of the total protein in the test diets, which were fed in amounts to maintain body weight.

Each food, or food combination, was studied at different levels of nitrogen intake in order to define the relationship between intake and balance. This relationship was shown to be rectilinear and satisfactorily described by the equation of a straight line in which nitrogen balance is the dependent variable (Y). The slope of the line, the *b* constant in the equation, is the product of the true digestibility of the dietary nitrogen and its biological value in the Thomas sense. The intercept of the line on the ordinate representing zero balance gives the amount of dietary nitrogen required for equilibrium. In these computations, all values for the different subjects are pooled together for each

test food by expressing them in milligrams of nitrogen per calorie of basal heat.

For milk, white flour, soy flour, the soy-white flour combination and the mixed foods, the indicated biological values were, respectively, 74, 41, 65, 55 and 65, and the amounts of nitrogen required for equilibrium (as ordinarily measured) were, respectively, 2.76, 4.76, 2.88, 3.38, and 3.12 mg. per basal calorie.

When due allowance is made for probable dermal losses of nitrogen and for the growth of tissues during adult life, the average daily requirements of conventional protein ($N \times 6.25$) calculated to a weight of 70 kg., a surface area of 1.8 square meters and a basal metabolism of 1650 cal. daily, are: 43 gm. for milk, 74 gm. for white flour, 47 gm. for soy flour, 54 gm. for the soy-white flour combination, and 50 gm. for the mixed foods. The limited significance of these numerical values is pointed out.

Some evidence is presented that the human adult, unlike the adult rat, requires lysine for nitrogen equilibrium.

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THE EFFECTS OF LIGHT, TRAUMA, RIBOFLAVIN, AND ARIBOFLAVINOSIS ON THE PRODUCTION OF CORNEAL VASCULARITY AND ON HEALING OF CORNEAL LESIONS [1]

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The growth of blood capillaries from the limbic plexus into the cornea has been observed as a response of this normally avascular tissue to a variety of adverse conditions (Duke-Elder, '42; Wolbach and Bessey, '42; Nutrition Reviews, '43); i.e., physical and chemical trauma, infections, and certain experimental dietary deficiencies. Among the dietary deficiencies, corneal vascularity has been observed in experimental vitamin A deficiency, ariboflavinosis, and in deficiencies of sodium, zinc, and the amino acids lysine and tryptophane. It has been clearly established that in man, just as in experimental animals, a deficiency of riboflavin may lead to an invasion of the cornea by capillaries (Sydenstricker, Sebrell, Cleckley and Kruse, '40). As a result, some investigators have attributed the mild degree of corneal vascularity found among apparently normal population groups, as well as among less well nourished groups, to riboflavin deficiency (Wiehl and Kruse, '41; Tisdall, McCreary and Pearce, '43; National Research Council, '43). This mild lesion has been found to be highly prevalent, particularly among sailors, aviators, and others exposed as a result of their activities to excessive sunlight; e.g., at high altitudes, on the desert, or from snow reflection of light or reflection from the water (unpublished confidential information). Since riboflavin is rapidly destroyed by light, the possibility exists that the exposure of the eye to bright light might further limit the riboflavin in the cornea and thereby accentuate the growth of capillaries into this structure. Bright light might even induce

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Public Health Research Institute of The City of New York, Inc.

a local riboflavin deficiency in the cornea in spite of an adequate intake of the vitamin for the rest of the body.

Trauma, if severe enough, also induces the growth of vessels into the cornea (Duke-Elder, '42). Ariboflavinosis might enhance this invasion of the cornea or delay recovery from the trauma. It is a common clinical experience that injuries to the cornea heal more quickly if the eye is bound so as to exclude light. It is conceivable that this benefit obtains through protection of the corneal riboflavin from destruction by light. If this were the case, a high riboflavin intake would be expected to overcome some of the detrimental effects of bright light on the healing of corneal injuries.

To study these effects and possibilities, an investigation has been made of the influence of light on the development of corneal lesions in riboflavin deficiency, and the influence of light and riboflavin on the rate of healing of injuries to the cornea. Three types of experiments have been made:

1. The rate of capillary ingrowth and other corneal changes have been compared in four groups of rats maintained under the following conditions: (a) Normal diet, kept in the dark; (b) normal diet, kept in continuous light; (c) riboflavin deficient diet, kept in the dark; (d) riboflavin deficient diet, kept in continuous light.

2. The rate of repair of injuries to the cornea produced by ultraviolet light, removal of the corneal epithelium, or by nitric acid has been assessed in groups of rats kept under the following conditions: (a) Normal diet, kept in the light; (b) normal diet, kept in the dark; (c) riboflavin deficient diet, kept in the light; (d) riboflavin deficient diet, kept in the dark; (e) normal diet plus extra riboflavin, kept in the light; (f) normal diet plus extra riboflavin, kept in the dark.

3. Observations have been made on the effects of feeding extra riboflavin on the prevalence of spontaneous corneal vascularity which regularly occurs in supposedly normal stock rats.

EXPERIMENTAL

Albino rats of the Wistar strain, 45 to 60 gm. in weight at the start of the experiment, were used in all experiments except for the ultraviolet irradiation injury; in this case the animals weighed 125–150 gm. The diet, supplements, and method for maintaining the animals in continuous light have been previously described (Bessey and Lowry, '44). Observations of the cornea were made with a binocular dissecting microscope using 3 × objectives and 10 × oculars. The cornea was illuminated with a 16 amp. 6-volt Mazda microscope illuminator lamp with

the image of the filament focussed on the eye ball. The light beam was allowed to pass through a nickel chloride solution to remove most of the heat. The beam of light was horizontal and the limbus was observed from above. By turning the eye so that it looked directly at the light, and allowing the light to fall on the lower portion of the cornea, the eye ball was filled with light and the limbic capillaries became easily visible against a brilliant white background. Small rats were held unanesthetized in the proper position with the eye ball protruding through tension with the fingers on either side of the eye. Larger rats were lightly anesthetized with ether. In some cases the limbic vessels were more easily observed after dilatation of the pupil with 2% homatropine. The normal vessels which extend only 0.25 to 0.4 mm. into the cornea were, however, more easily observed without mydriasis. The length of the capillaries was measured by comparison with the separation between hairs placed in the ocular of the microscope. The vessels were in some instances photographed by mounting a camera (Loveland, '43) over one eye piece of the binocular microscope and judging the focus through the other eye piece. When the rat was quiet and the vessels exactly in focus, the camera shutter was tripped with a foot attachment.

The three types of trauma used in studying the corneal response to injury were produced in the following way: The corneal epithelium was removed with sand paper. It was difficult to avoid some injury to the underlying stroma as well. With damage to the stroma, healing was greatly delayed. This introduced a factor difficult to control. The trauma from acid was produced by applying nitric acid, 1N or 5N, for a definite length of time with a wooden applicator which had been reduced to about ½ mm. in diameter and which was provided with a thin cotton wrapping to give a total diameter of about 1 mm. By dipping this applicator in the diluted nitric acid and applying it at right angles to the cornea, a damaged area 1 mm. in diameter could be produced. The irradiation with ultraviolet light was accomplished by anesthetizing the rats with Nembutal, binding one eye open with adhesive tape and exposing the open eye to the light from a General Electric H4 mercury vapor lamp. The head of the rat was bound to a board with a strip of adhesive tape held in place with thumb tacks. In this way the cornea was kept at a standard distance (5 cm.) from the envelope of the lamp. Of the three types of trauma produced, this last was the most reproducible.

The changes resulting from riboflavin deficiency were grouped according to (a) length of corneal capillaries; (b) alteration of the capillaries, i.e., enlargement, bulging and sprouting; (c) turbidity of the cornea,

and (d) swelling and redness of the conjunctiva. The changes, other than change in length of capillaries, were assigned numerical values as per cent of the severest changes observed; e.g., slight, moderate, severe, and extreme changes were recorded as 25, 50, 75, and 100%, respectively. The values for "capillary alteration" represent the average of observed values for enlargement, bulging and sprouting. In the case of trauma to the eye, the changes were assessed in an analogous manner, complete healing of the cornea being considered as 100% recovery.

RESULTS AND DISCUSSION

Influence of light on the corneal vascularization of ariboflavinosis

In general, long exposure to light from incandescent lamps failed to accelerate the invasion of the cornea by blood vessels in ariboflavinosis. At the end of 28 days (table 1) rats on a riboflavin-free diet were no less severely affected if maintained in darkness than if kept in bright light.

In the normal rat, capillaries extend 0.25 to 0.4 mm. into the cornea from the limbic vessels. In the event of severe riboflavin deficiency, the capillaries will grow to many times their initial length. It is unusual to

TABLE 1

Observation of eyes after 28 days on experiment.

DIET	ENVIRONMENT	NO. OF ANIMALS	INITIAL WEIGHT	GAIN IN 28 DAYS	CORNEA			CONJUNCTIVA
					Capillary length	Capillary alteration	Turbidity	Swelling and redness
			gm.	*gm.*	*mm.*	*% of maximal* [1]		
Riboflavin deficient	Dark	8	58	8	0.9	45	38	50
Riboflavin deficient	Bright light	7	55	19	0.6	20	22	50
Normal control 20 μg. riboflavin daily	Dark	6	60	30	0.4	2	0	15
Normal control 20 μg. riboflavin daily	Bright light	7	55	55	0.4	7	2	20

[1] The derivation of these average percentage figures is based on an arbitrary percentage scale and these values should not be taken to indicate that the measurements could be made with great precision (see text).

observe growth limited to 0.1 or 0.2 mm. Thus, the average capillary lengths in the deficient corneas (table 1) represent the mean of a number of 0.3 or 0.4 mm. values with other values of 0.8 to 2 mm. In the control group no influence of light on the eyes could be observed at the end of 28 days, although early in the experiment the rats exposed to the brilliant illumination showed a moderate degree of reddening and swelling of the conjunctivae. Both groups of rats gained less weight in darkness than they did in the light. This depressant effect of nearly complete and continuous darkness has been observed previously (Dempsey). This growth effect appears to be without significance for the present studies since another group of rats maintained in moderate light developed eye changes on a riboflavin-free diet which were approximately the same as those shown in table 1.

These observations are in agreement with chemical analyses (Bessey and Lowry, '44) which demonstrated that light did not influence the concentration of riboflavin in the cornea. The chemical studies also showed that corneal changes were not visible until the riboflavin concentration in the cornea had fallen to less than half of normal. This would seem to indicate that the corneal changes are evidence of rather extreme depletion of riboflavin. This conclusion is supported by the prompt improvement of the cornea with very small doses of riboflavin.

Influence of light and riboflavin on the healing of corneal injuries

Exposure to ultraviolet light proved to be the most satisfactory form of trauma from the standpoint of reproducibility. The healing ability of the cornea was measured by the length of exposure which on repetition at 24-hour intervals would just produce mild but detectable changes in the cornea. The exposures were repeated as many as five or six times. The time of exposure required was reproducible within 15 or 20%.

With this method it could be easily shown (table 2) that (a) the cornea can tolerate more ultraviolet light if the intervals between exposures are spent in darkness; (b) that neither extra riboflavin nor 2 weeks' deprivation of riboflavin has demonstrable influence on the response to ultraviolet light; but (c) that riboflavin deprivation for longer periods in either the young or the adult does handicap the cornea in resisting trauma due to ultraviolet light, particularly if capillaries are already actively growing in the cornea.

Although the striking delay in the rate of healing due to light in all groups is consistent with the thesis that light might handicap the cornea by the local destruction of riboflavin, the chemical analyses of the cornea have failed to support this possibility (Bessey and Lowry, '44). Not

only did ultraviolet illumination alone, or exposure to incandescent lamps alone fail to decrease the corneal riboflavin concentration, but the combination of the two types of illumination was equally ineffective in lowering the riboflavin in the cornea. Although light exerts a striking effect, this does not seem explicable on the basis of riboflavin destruction. These observations indicate that although mild riboflavin deficiency does not lead to handicaps in healing of corneal injuries due to light, there is a degree of deficiency beyond which very definite handicaps result. Whether riboflavin deficiency is specific in this respect, or whether it is a response common to other deficiencies, cannot be decided

TABLE 2

Time of exposure to ultraviolet light required to produce definite corneal changes.

		NORMAL ADULTS FED		ADULTS RIBOFLAVIN DEFICIENT FOR		YOUNG RATS RIBOFLAVIN DEFICIENT	
		Normal diets [1]	Normal diets plus riboflavin [2]	17 days [3]	30 days	Quiescent capillaries	Growing capillaries
Rats kept in darkness between irradiation	Minutes required	28	28	25	15	18	12
	Number of animals	(19)	(6)	(5)	(4)	(4)	(2)
Rats kept in light between irradiation	Minutes required	10	10	10	..	..	..
	Number of animals	(8)	(4)	(4)	..	..	.

[1] Rats either on chow (3 μg. per gm. of diet) or purified diet plus 21 μg. riboflavin per day.
[2] Forty μg. extra riboflavin daily.
[3] No corneal changes visible before irradiation.

without further studies. It does seem clear, however, that the effects from light and the effects from riboflavin deficiency are independent factors.

The other two types of corneal trauma employed, mechanical injury by removal of the epithelium and chemical injury with nitric acid, proved to be less uniform and less sensitive methods (table 3). Neither the light in itself nor the riboflavin deficiency alone retarded healing to a degree detectable by these methods. However, the combination of light after injury and riboflavin deficiency did lead to striking handicaps. The lack of sensitivity of these later methods may possibly be ascribed to the surface nature of the injuries compared with the presumably more penetrating characteristics of the injury caused by ultraviolet light. However, the possibility must be considered that light may

TABLE 3

Per cent recovery[1] *of cornea 5 to 7 days after trauma.*

	ADULTS KEPT IN DARK			ADULTS KEPT IN LIGHT		
	Normal[2]	Normal + riboflavin[3]	Riboflavin deficient[4]	Normal[2]	Normal + riboflavin[3]	Riboflavin deficient[4]
Removal of epithelium	60	68	68	50	68	0
Number of animals	(6)	(3)	(3)	(6)	(3)	(3)
5N HNO_3 for 15 seconds	52	32	50	72	50	18
Number of animals	(6)	(3)	(3)	(6)	(3)	(3)

[1] The derivation of these average percentage figures is based on an arbitrary percentage scale and these values should not be taken to indicate that the measurements could be made with great precision (see text).

[2] Three μg. riboflavin per gram of diet (chow) or about 30 μg. daily.

[3] Forty μg. extra riboflavin per day or a total of about 70 μg. daily.

[4] Thirty days on riboflavin deficient diet.

be more damaging to an injury produced by light than to other types of injury.

Influence of riboflavin on the development of "spontaneous" capillaries

Older rats on a dog chow diet frequently develop corneal vascularity which may occasionally approach the severity of invasion observed in riboflavin deficiency. To test the influence of added riboflavin on this "spontaneous" corneal vascularity, a group of twenty rats were put on a chow diet at the time of weaning. Six of these rats were given an extra supplement of 35 μg. of riboflavin per day. At the end of 66 days the average capillary length in the control group was 0.30 mm. (S.E. = .02 mm.) and in the group receiving extra riboflavin 0.35 mm. (S.E. = .07 mm.). By the end of 131 days some of the rats in both groups had corneal vessels definitely longer than "normal". These raised the average capillary length to 0.5 mm. (S.E. = .07 mm.) in the control group and 0.64 (S.E. = .09 mm.) in the group with added riboflavin.

The failure of added riboflavin to influence the incidence of "spontaneous" capillaries in these rats as well as the proof that corneal riboflavin is not affected by ultraviolet light in spite of capillary invasion therefrom, re-emphasizes the fact that riboflavin deficiency is only one of a number of conditions which will lead to corneal vascularization.

SUMMARY AND CONCLUSIONS

1. Brilliant continuous illumination with incandescent lamps did not augment the changes in the cornea and conjunctiva resulting from riboflavin deficiency.

2. The healing of injury of the cornea resulting from ultraviolet light was handicapped by brilliant continuous illumination from incandescent lamps and by protracted riboflavin deficiency. However, the failure to observe an effect of either visible or ultraviolet light on the riboflavin content of the cornea indicates that this handicap is not the result of local destruction of riboflavin by light but due to some other cause. The healing of injury due to milder trauma, that involving primarily the epithelium, was not affected by either light or riboflavin deficiency alone under the conditions of these experiments. However, a combination of these two factors did lead to definite handicaps.

3. High riboflavin intake failed to prevent the occurrence of "spontaneous corneal vascularity" occurring in a normally nourished rat colony.

4. The variety of agents and deficiencies which will induce corneal vascularization indicates the need for caution in interpretation of corneal vascularization in man.

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COMPARATIVE EFFECTIVENESS OF VARIOUS IRON COMPOUNDS IN PROMOTING IRON RETENTION AND HEMOGLOBIN REGENERATION BY ANEMIC RATS

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Iron compounds used for the enrichment of bread and flour should possess two qualifications. First they should be utilized by the body and secondly, they should have no adverse effect upon the preservation of flour (Fed. Reg., '41). Most compounds of iron that are readily soluble in water and dilute acid cause flour to become rancid or to have a decreased vitamin content (Gillet, '45). Sodium iron pyrophosphate seems to have no adverse effect on flour, but there is some difference of opinion as to the effectiveness of its utilization by the body. Nakamura and Mitchell ('43) reported a relatively high degree of utilization of this compound in anemic rats while Street ('43) found it to have only about half the effectiveness of ferrous sulphate in promoting hemoglobin regeneration in anemic rats.

The present study was undertaken to compare various iron compounds used for the enrichment of bread and flour as to their effect on iron retention and hemoglobin regeneration in anemic rats. The compounds were not only tested as such, but were also compared when used as the fortifying ingredient in specially prepared breads. Some observations were also made on the efficacy of two iron compounds in preventing anemia in milk-fed rats.

METHODS

Hemoglobin was determined by the method of Wu ('22) as adapted to a photoelectric colorimeter. The white blood cell pipettes used for hemoglobin estimations were calibrated on a standard blood of known iron content. The iron analyses by the thiocyanate method were done as previously described (Freeman and Ivy, '42) with only one modification. Immediately after extraction of the iron thiocyanate with iso-

amyl alcohol, the colorimeter tubes containing the extract were warmed in a water bath at 40°C. for 5 minutes and then read.

The rats used in the experiment were distributed among the various groups according to weight and sex so as to make the groups as uniform and comparable as possible.

Anemia was produced according to the method of Elvehjem and Kemmerer ('31) using cages that were described previously (Freeman and Ivy, '42). The rats were depleted on a milk diet until the hemoglobin concentration was approximately 3.0 gm./100 ml. of blood. This degree of depletion usually required 35 to 45 days after weaning (21 days of age).

The depleted rats received the iron compounds under study as a supplement to the milk diet which was offered ad libitum. The iron compounds were mixed with cane sugar in such proportion that 1 gm. of the mixture contained 0.25 mg. of iron. The iron content of the mixture was verified by analysis. That amount of iron-sugar mixture providing 0.25 mg. of iron daily for 28 days (as determined by analysis) was divided into 28 capsules (no. 000). The contents of one capsule were fed daily in a clean salt cellar with added thiamine chloride (10 γ) and copper and manganese as sulphates (0.05 mg. of each). The daily supply of milk was withheld until the supplement was consumed.

The breads containing the various iron compounds were made from a dough of the following composition:[1] flour (unenriched) 100; water 65; yeast 2; salt 2; sugar 5; milk (dried skim) 3; yeast food 0.5; and lard 2.

To this basic mixture was added an amount of iron which would give 0.20 mg. of extra iron to each 5 gm. of air-dried bread or 18 mg. of extra iron per loaf of bread.[2] The iron salt was thoroughly mixed with the dry ingredients before the dough was prepared. The baked bread was sliced, air-dried, ground, mixed and analyzed. According to the analyses, the amount of dried ground bread which contained 0.27 mg. of iron was 4.5 to 5.0 gm. This amount of bread was fed daily to the experimental animals. The control rats received the same amount of the plain bread which contained 0.07 mg. of iron by analysis. Milk was offered to the animals only after the bread was completely consumed.

The rats were killed after 28 days on the supplemented diet. Hemoglobin determinations were made on the seventh, seventeenth and twenty-eighth days.

[1] Prepared by the American Institute of Baking.

[2] This amount of iron is slightly in excess of that recommended for the enrichment of bread and flour by the National Research Council. See Bull. Nat. Res. Council, no. 110, Nov. 1944, "Enrichment of Flour and Bread".

In the part of the study concerning prevention of anemia in milk-fed rats by iron compounds, the weanling rats (21 days old) were given 0.25 mg. of iron daily either as ferric chloride or sodium iron pyrophosphate in a sugar mixture similar to that described above. The control group received the sugar alone. Each day, after the sugar or sugar mixture was entirely consumed, the rats were given milk ad libitum. Hemoglobin values were determined at 15, 30 and 40 days, At 40 days the rats were killed and the carcasses analyzed for iron content. Hemoglobin and iron determinations were also made on 21-day-old rats that only had access to milk since the twelfth day of life.

RESULTS

The iron content of the carcass after 40 days of depletion was found to average 0.94 mg. per rat (see table 2). This value was taken as the iron content of all the depleted rats used in this study and the retention of iron from any supplement was calculated by subtracting 0.94 from the final iron content of the carcass. The total iron intake from bread or supplement divided into the iron retained by rats on the supplement times 100 gives the percentage retention from various sources. The relative iron retention was calculated by comparing retention from other sources with that from ferric chloride. The high relative retention of the iron contained in plain bread is in accord with the finding of Smith and Otis ('37), who showed that small amounts of iron result in a relatively greater hemoglobin regeneration by anemic rats. According to these same authors, the total daily amount of iron fed in these experiments is at the upper limits of the range over which there is a direct relation between hemoglobin regeneration and iron intake. The data reported here demonstrate a direct relation between iron retention and hemoglobin regeneration.

Iron retention and hemoglobin regeneration by anemic rats on the various iron salts when fed as such or contained in bread are presented in table 1. These data show a good correlation between hemoglobin concentration and the iron content of the carcass.

The rats which received ferric chloride with sugar or in bread showed the greatest iron retention and hemoglobin formation.

Iron retention and hemoglobin regeneration for sodium ferric orthophosphate, both as the salt and in bread, and for ferric orthophosphate in bread were only slightly less than for ferric chloride iron. Reduced iron was somewhat less effective, both as the salt and in bread. Sodium iron pyrophosphate was the least effective of the compounds studied. Doubling the daily intake of this salt was without significant effect upon iron retention or hemoglobin regeneration.

TABLE 1

Iron retention and hemoglobin formation by anemic rats receiving iron supplements or iron enriched bread.

GROUP NO.	SOURCE OF Fe [1]	NO. RATS IN GROUP	AVE. PERIOD OF DEPLETION	AVE. WT. AT START OF SUPPLEMENT	AVE. WT. GAIN ON SUPPLEMENT	AVE. Hb AT START OF SUPPLEMENT	AVE. Hb INCREASE ON SUPPLEMENT		AVE. TOTAL Fe CONTENT OF CARCASS		RELATIVE Hb REGENERATION IN %	RELATIVE RETENTION OF Fe	% Fe SUPPLEMENT RETAINED
			days	*gm.*	*gm.*	*gm./100 ml.*	*gm./100 ml.*	C_1 [2]	*mg.*	C_1 [2]		%	
1	Ferric chloride	10	34	83	102	3.07	10.65		5.83				
2	Ferric chloride	10	36	89	107	2.96 } 3.01	9.72 } 10.00 ± 1.17		5.60 } 5.75 ± 0.70		100	100	68.0
3	Ferric chloride	9	38	93	91	3.00	9.47		5.82				
4	Sodium iron pyrophosphate	10	35	85	92	3.04	4.76 ± 1.74	2.5	3.19 ± 0.42	3.7	48.0	46.6	32.1
5	"Double amount" sodium iron pyrophosphate	6	36	90	55	2.97	6.18 ± 1.50	2.5	2.91 ± 0.13	3.8	62.3	40.9	14.0
6	Reduced iron	10	34	84	107	2.92	8.85 ± 0.89	0.8	5.15 ± 0.59	0.6	83.4	87.5	59.3
7	Sodium ferric orthophosphate	10	39	94	88	2.97	9.26 ± 1.52	0.4	5.26 ± 0.59	0.5	93.5	89.9	61.0
8	$FeCl_3$ bread	10	39	94	86	2.92	9.28 ± 1.10		5.61 ± 0.62		100	100	61.0
9	Sodium iron pyrophosphate bread	10	34	91	83	3.00	4.09 ± 1.10	3.5	3.32 ± 0.69	2.7	44.5	51.0	30.8
10	Reduced iron bread	9	44	91	93	2.78	7.95 ± 1.64	0.7	4.86 ± 1.00	0.5	86.0	84.0	51.0
11	Ferric orthophosphate bread	9	42	92	107	3.06	8.30 ± 1.48	0.5	5.27 ± 0.74	0.4	90.5	92.7	56.5
12	Sodium ferric orthophosphate bread	9	45	94	98	2.86	8.66 ± 1.13	0.5	5.29 ± 0.73	0.3	93.2	93.1	56.7
13	Plain bread	11	40	92	69	2.92	2.38 ± 1.09	4.9	2.33 ± 0.36	6.4	25.9	29.8	68.0

[1] Source of iron compounds (1, 2, 3, 8) Mallinckrodt, (4, 5, 9, 12) Victor Chemical, (6, 10, 11) Merck & Co.

[2] C_1 = C.R. = critical ratio, considered significant when greater than 2 according to Fischer's rule.

There is good agreement between the results obtained when the iron salts were fed mixed with sugar and when contained in bread. The relative retention of iron from various sources is in the same order in either case. The absolute amounts retained from any given compound are so similar for iron contained in bread as compared to that mixed with sugar as to indicate that the absorption of iron was not significantly altered by its inclusion in the bread. Widdowson and McCance ('42) found that iron was absorbed by human subjects from a diet that contained 40–50% of its calories as white bread but that its absorption was reduced when white flour was replaced by one containing considerable quantities of bran.

TABLE 2

Prevention of anemia in milk-fed rats.

SUPPLEMENT	NO. RATS IN GROUP	TIME ON EXPERIMENT	INITIAL WT.	AVE. WT. GAIN ON SUPPLEMENT	AVE. Hb AT 15 DAYS OF SUPPLEMENT	AVERAGE FINAL Hb	AVE. Fe CONTENT OF CARCASS	Fe RETAINED [2]	RELATIVE RETENTION OF Fe	RELATIVE INCREASE IN HEMOGLOBIN
		days	*gm.*		*gm./100 ml.*	*gm./100 ml.*	*mg.*	*mg.*	%	%
$FeCl_3$ + sugar	7	40	30	62	13.60	13.08 ± 2.03	4.00 ± 0.97	3.06	100	100
Pyro [1] + sugar	7	40	28	80	7.87	7.20 ± 0.66	2.32 ± 0.3	1.38	45	41.5
Sugar	6	40	28	44	6.12	3.04 ± 0.72	0.943 ± 0.21			
21-day-old rats	12	0	35			9.40 ± 0.33	1.06 ± 0.14			

[1] Sodium iron pyrophosphate.

[2] Final Fe content of Fe supplemented group minus Fe content of group fed sugar alone.

A lower iron retention and hemoglobin regeneration by anemic rats fed sodium iron pyrophosphate was demonstrated in three separate experiments; first, with the compound fed at two concentrations, second, when added to bread and third when it failed to prevent the development of an anemia in rats (table 2). It does provide sufficient iron to permit growth but the retention of iron from this source was only approximately half that of ferric chloride, whether used in the treatment or prevention of anemia.

The various iron compounds show the following order of effectiveness in their relative retention by anemic rats: ferric chloride > sodium

ferric orthophosphate = ferric orthophosphate > reduced iron > sodium iron pyrophosphate (table 1). The order is the same whether the rats were fed the compounds themselves or received bread containing them. The relative degrees of hemoglobin regeneration for the compounds or enriched breads also give the same order whether determined after 7, 17 or 28 days. For this reason only the final hemoglobin values are included in the table.

Street ('43) studied hemoglobin regeneration in anemic rats and obtained results which indicate essentially the same relative utilization of ferrous sulfate and sodium iron pyrophosphate as that which we have obtained for ferric chloride and sodium iron pyrophosphate. The higher hemoglobin concentration on sodium iron pyrophosphate reported by Nakamura and Mitchell ('43) may be due to the relatively low weight gain of their rats during the experimental period. The degree of anemia was also less in their animals at the beginning of the experimental period. Iron retention was greater in 21 days for three sodium iron pyrophosphate rats reported by Nakamura and Mitchell than for our 28-day animals maintained on the same supplement, while ferric chloride retention was relatively greater in our animals.

The uniformity of iron retention and hemoglobin regeneration by anemic rats on a given compound is illustrated by the data obtained on three different groups of rats fed ferric chloride. These three groups were controls for rats fed sodium iron pyrophosphate, reduced iron and sodium ferric orthophosphate. The three groups were studied at different times and the rats were from different litters. There is good agreement among the three groups both as to iron retention and hemoglobin regeneration. Variation in hemoglobin regeneration is greater than for iron retention but the hemoglobin increase is also greater so that the impression derived from either determination is generally much the same. In studying the efficacy of a given iron compound as a source of iron, iron content appears to be a more direct measure than hemoglobin concentration. Other factors than iron absorption may affect hemoglobin regeneration and the concentration of hemoglobin in the blood is subjected to other factors that influence blood volume. The influence of growth on the concentration of hemoglobin in animals with a similar iron retention is well illustrated by groups 4 and 5, fed different amounts of sodium iron pyrophosphate (table 1). Although iron retention was essentially the same for the two groups, the hemoglobin increase of group 5 was on an average about 2 gm. higher while the weight gain of this group was only slightly more than half that of group 4.

In the prevention of anemia in weanling rats, the relative iron retention of ferric chloride and sodium iron pyrophosphate was similar to that obtained in the depleted rats (see table 2). So far as hemoglobin formation is concerned, this experiment is theoretically complicated by the fact that the diet is deficient in copper and manganese. If these two substances are supplied the development of anemia may be retarded, while if these substances are not supplied hemoglobin formation may be influenced by their absence as well as by the availability of iron. At the end of this experiment the variability both of hemoglobin and total iron content was greater for the rats fed ferric chloride than was the case at the end of the depletion experiments (see table 1).

The prevention of anemia in milk-fed rats offers a method of evaluating iron compounds which has certain desirable aspects. This procedure saves time since the experiment is ended by the time the control animals are depleted which is actually the starting point in the depletion experiments. Thus the prevention method covers a span of 40 days' time while the depletion method in our experience takes 60 to 70 days. During depletion some rats do not grow sufficiently or develop respiratory tract infections and have to be discarded as unfit for experimental material. This loss, representing considerable wasted time and effort, is reduced when the source of iron is fed from the time of weaning, only the control group being subjected to these hazards. The hemoglobin and total iron content of the twelve rats killed at weaning in the present experiment (table 2) indicate that the rats at this age were quite uniform when treated as described by Elvehjem and Kemmerer ('31).

SUMMARY

1. The retention of iron from different sources by anemic rats was qualitatively and quantitatively similar irrespective of whether the iron salts were fed as such or contained in bread.

2. The various iron compounds tested showed the following order of effectiveness with respect to the relative degree of iron retention and hemoglobin regeneration produced in anemic rats: ferric chloride > sodium ferric orthophosphate = ferric phosphate > reduced iron > sodium iron pyrophosphate.

3. Prevention of anemia in milk-fed rats given supplements of ferric chloride or sodium iron pyrophasphate for 40 days after weaning (21 days) gave results for relative iron retention and hemoglobin regeneration similar to those obtained with depleted rats fed the same supplements for 28 days.

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